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(54) Title: MINIMALLY IMMUNOGENIC VARIANTS OF HUMANIZED COL-1 ANTIBODY AGAINST CARCINOEMBRYONIC ANTIGEN

(57) Abstract: The present disclosure provides humanized COL-1 monoclonal antibodies that are minimally immunogenic and that retain carcinoembryonic antigen binding affinity. In one embodiment, a humanized COL-1 antibody includes an amino acid substitution at position 61 in a heavy chain complementarity determining region 2 of the murine COL-1 antibody. In another embodiment, a humanized COL-1 antibody includes an amino acid substitution at position 24, 25, and 27 in a light chain complementarity determining region 1 of the murine COL-1 antibody. In several embodiments, methods are disclosed for the use of a humanized COL-1 antibody in the detection or treatment of a tumor in a subject. Also disclosed is a kit including the humanized COL-1 antibody described herein.

MINIMALLY IMMUNOGENIC VARIANTS OF HUMANIZED COL-1 ANTIBODY AGAINST CARCINOEMBRYONIC ANTIGEN

PRIORITY CLAIM

This application claims the benefit of U.S. Provisional Application No. 60/408,703, filed September 5, 2002, which is incorporated herein by reference.

FIELD

The present disclosure relates to humanized monoclonal antibodies that bind a tumor antigen. More specifically, the present disclosure relates to humanized monoclonal antibodies with amino acid substitutions that have minimal immunogenicity and retain antigen binding affinity for the carcinoembryonic antigen (CEA).

BACKGROUND

Monoclonal antibodies (mAbs) raised against tumor antigens hold promise for diagnosis and therapy of human cancers (reviewed in: Weiner, *Semin. Oncol.* 26: 43, 1999; Green *et al., Cancer Treat. Rev.* 26:269, 2000; Carter, *Nature Rev.* 1:118, 2001). A major impediment to the clinical use of murine mAbs is the human anti-murine antibody (HAMA) response these mAbs elicit in patients (Seccamani *et al., Int. J. Rad. Appl. Instrum. B.* 16:167, 1989; Reynolds *et al., Int. J. Rad. Appl. Instrum. B.* 16:121, 1989; Colcher *et al., J. Nucl. Med.* 31:1133, 1990; Blanco *et al., J Clin. Immunol.* 17:96, 1997). To obviate the potential HAMA response, humanized Abs have been developed by grafting the complementarity determining regions (CDRs) of the murine Abs onto the frameworks of the variable light (V_L) and variable heavy (V_H) regions of human mAbs (reviewed in Winter and Harris. *Immunol. Today* 14:243, 1993). Humanization of a xenogenic antibody, however, does not necessarily eliminate the immunogenicity of the molecule since the humanized molecule can evoke anti-V region response (Blanco *et al., J Clin. Immunol.* 17:96, 1997; Schneider *et al., J. Immunol.* 150:3086, 1993; Stephens *et al., Immunology* 85:668, 1995; Sharkey *et al. Cancer Res.*

55: 5935s, 1995; Iwahashi *et al., Mol. Immunol.* 36:1079, 1999; Tamura *et al., J. Immunol.* 164:1432, 2000).

CEA is a member of the immunoglobulin superfamily that includes normal fecal antigen, non-specific cross-reacting antigen, meconium antigen, and biliary glycoprotein. It is composed of seven domains linked to the cell membrane through a glycosylphosphatidylinositol anchor and has a molecular weight of 180 kDa. CEA is normally expressed in a variety of glandular epithelial tissues where it appears to be localized to the apical surface of the cells, although it is expressed in numerous carcinomas including gastrointestinal, colorectal, breast, ovarian and lung carcinomas. CEA is an especially well characterized human tumor antigen and is widely used for the diagnosis of human colon cancer.

Monoclonal antibodies have been generated to detect various epitopes on CEA, and in using these antibodies the differential expression of CEA has been determined (Muraro *et al., Cancer Res.*, 45:5769, 1985; Ohuchi *et al., Cancer Res.* 47:3565, 1987; Wilkinson *et al., Proc. Natl. Acad. Sci.* 98:10256, 2001). Of these monoclonal antibodies, COL-1 is of clinical importance because it has a high affinity for CEA. In addition, COL-1 reacts specifically with CEA and not with CEA-related antigens such as normal fecal antigen and non-specific cross-reacting antigen (Kuroki *et al., Int. J. Cancer* 44:208, 1989; Robbins *et al., Int. J. Cancer* 53:892, 1993). As a result of these properties, radiolabeled COL-1 has been used as a therapeutic agent in the treatment of patients with tumors that express CEA. Unfortunately, the murine origin of the antibody results in a HAMA response in these patients. Thus, there clearly exists a need to develop a humanized COL-1 antibody with minimal immunogenicity but that does not suffer from a loss in antigen binding affinity.

SUMMARY

The present disclosure relates to humanized COL-1 monoclonal antibodies that are minimally immunogenic and that retain CEA binding affinity.

In one embodiment, a humanized COL-1 monoclonal antibody is disclosed that includes a light chain Complementarity Determining Region (LCDR)1, a LCDR2, and a

LCDR3; and a heavy chain Complementarity Determining Region (HCDR)1, a HCDR2, and a HCDR3 from monoclonal antibody COL-1, wherein the LCDR1, LCDR2, LCDR3 are in a human light chain framework and wherein the LCDR1, LCDR-2 and LCDR3 are in a human heavy chain framework. The humanized COL-1 monoclonal antibody also includes an amino acid substitution of a non-ligand contact residue in HCDR2, for example at position 61. It is shown herein that the humanized COL-1 retains, or has increased, binding affinity for CEA and has reduced immunogenicity, as compared to a parental antibody.

In another embodiment, a humanized COL-1 antibody is disclosed that is encoded by the nucleic acid sequence deposited as ATCC Accession number PTA-4642, ATCC Accession number PTA-4643, or ATCC Accession number PTA-4644 (Deposited September 5, 2002).

In another embodiment, a humanized COL-1 monoclonal antibody is disclosed that includes an amino acid substitution at position 24, 25, or 27 in LCDR1 of a murine COL-1 antibody and is minimally immunogenic in a subject.

Methods are disclosed for the use of the humanized COL-1 monoclonal antibodies disclosed herein. A kit including the antibodies disclosed herein is also described.

The foregoing and other features and advantages will become more apparent from the following detailed description of several embodiments, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A-B are schematic representations of the nucleotide sequence of the genes encoding the V regions of HuCOL-1 and their leader peptides. Nucleotide sequences of the humanized V_L (FIG. 1A; SEQ ID NO: 11) and V_H (FIG. 1B; SEQ ID NO: 12) genes were generated and amplified by PCR amplification, using four overlapping synthetic oligonucleotides (indicated by arrows) that together encompass, on alternating strands, the entire sequence of each of the genes and its leader.

Sequences on the flanks of the genes encoding the variable region domains and their leader peptides are shown by lower case letters. The V_L region (FIG. 1A) is comprised of nucleotides from position 73 to 402, while the V_H region (FIG. 1B) starts from position 70 and ends at 441. The restriction enzyme sites incorporated in the oligomers to facilitate cloning are shown in italics.

FIG. 2A-B are schematic representations of the nucleotide and amino acid sequences of murine monoclonal antibody COL-1 (mCOL-1). Nucleotide sequences of the V_L (FIG. 2A; SEQ ID NO: 13) and V_H (FIG. 2B; SEQ ID NO: 14) regions of COL-1 were determined and the amino acid sequences of the V_L (SEQ ID NO: 15) and V_H (SEQ ID NO: 16) regions were deduced from the nucleotide sequences.

FIG. 3A-C are schematic representations of the humanization protocols for mAb COL-1. FIG. 3A shows the amino acid sequences of the V_L regions of murine COL-1 (SEQ ID NO: 15), human Ab VJI'CL (SEQ ID NO: 17), HuCOL-1 derived from mCOL-1 and VJI'CL (SEQ ID NO: 18), and the HuCOL-1 variant ^{24,25,27}L (SEQ ID NO: 19). FIG. 3B shows the amino acid sequences of the V_H regions of mCOL-1 (SEQ ID NO: 19), human antibody MO30 (SEQ ID NO: 20), HuCOL-1 derived from mCOL-1 and MO30 (SEQ ID NO: 21), and HuCOL-1 variant ^{61}H (SEQ ID NO: 22). Dashes indicate residues that are identical in mCOL-1, human and humanized antibodies. Asterisks mark frameworks residues that are deemed essential for maintaining the combining site structure of mCOL-1. Murine frameworks residues retained in the HuCOL-1 are shown in bold. FIG. 3C shows the amino acid sequences of CDR1 in the V_L regions of murine COL-1 (SEQ ID NO: 23), human Ab VJI'CL (SEQ ID NO: 24), HuCOL-1 derived from mCOL-1 and VJI'CL (SEQ ID NO: 25), and the HuCOL-1 variant ^{24,25,27}L (SEQ ID NO: 26) and the amino acid sequences of CDR2 in the V_H regions of mCOL-1 (SEQ ID NO: 27), human antibody MO30 (SEQ ID NO: 28), HuCOL-1 derived from mCOL-1 and MO30 (SEQ ID NO: 29), and HuCOL-1 variant ^{61}H (SEQ ID NO: 30). Dashes indicate residues that are identical between the

antibodies. Shaded areas show CDR residues that are substituted in HuCOL-$1^{24,25,27}$L, HuCOL-1^{61}H, and HuCOL-$1^{24,25,27}$L/^{61}H.

FIG. 4A-B are schematic representations of the dual expression constructs derived from the baculovirus vectors. Genes encoding the light (L) and heavy (H) chains of cCOL-1 were cloned in pAcUW51 (FIG. 4A) and those of HuCOL-1 or HuCOL-1 variants were cloned in pBACx-1 (FIG. 4B) vectors, downstream from the appropriate promoters. P10 and *polh* represent P10 and polyhedrin promoters; ori and f1 are SV40 and f1 origin of replication; Amp^R represents an ampicillin-resistant gene; arrows show the direction of transcription.

FIG. 5A-B are a set of digital images demonstrating SDS-PAGE analysis of the purified monoclonal Abs derived from murine COL-1. Samples of the monoclonal Abs were analyzed in non-reducing (FIG. 5A) and reducing (FIG. 5B) conditions. Lane 1, murine COL-1; lane 2, cCOL-1; lane 3, HuCOL-1; lanes 4-6, variants ^{24,25,27}L, ^{61}H and ^{24,25,27}L/^{61}H. Sizes of the molecular weight markers (BioRad, Hercules, CA) are given in the column at left.

FIG. 6A-B are a set of graphs demonstrating the reactivity of various murine COL-1-derived antibodies (identified by their symbols, below) in a competition RIA assay. Increasing concentrations of mAbs mCOL-1 (◆), cCOL-1 (■), HuCOL-1 (▲), ^{24,25,27}L (○), ^{61}H (□), ^{24,25,27}L/^{61}H (●) and HuIgG (△) were used to compete for the binding of ^{125}I-labeled mCOL-1 (FIG. 6A) and ^{125}I-labeled HuCOL-1 (FIG. 6B) to 200 ng of CEA coated in each well. Dashed lines indicate competitor murine COL-1 and HuCOL-1 in FIG. 6A and 6B, respectively.

FIG. 7A-H are a series of graphs demonstrating the flow cytometric analysis of the binding of HuCOL-1 and its variants to cells expressing cell surface CEA. Binding profiles of 1 μg (FIG. 7A-7D) and 0.5 μg (FIG.. 7E-7H) of HuCOL-1 (FIG. 7A and 7E), ^{24,25,27}L (FIG. 7B and 7F), ^{61}H (FIG. 7C and 7G), and ^{24,25,27}L/^{61}H (FIG. 7D and

7H) mAbs, respectively, to MC38 cells engineered to express CEA on its cell surface are shown. Binding of an irrelevant monoclonal Ab, human IgG (dashed line), is shown in each panel and represents less than 2% of the cell population.

FIG. 8 is a graph representing the binding of pre-adsorbed patients' sera JS, EM, MB, and serum from a normal donor to immobilized HuCOL-1. The sera were diluted (1:5) and applied, using an external pump, to the surfaces of the flow cells 1 and 2. Shown are response differences in the association phase between flow cell 1 (immobilized HuCOL-1) and flow cell 2 (immobilized control protein).

FIG. 9A-C are a series of graphs that represent sera reactivity, by SPR, of HuCOL-1 and its variants. Increasing concentrations of HuCOL-1 (▲), ^{24,25,27}L (○), ^{61}H (□), and ^{24,25,27}L/^{61}H (●) mAbs were used to compete with the anti-V region Abs to COL-1 present in sera from patients EM (FIG. 9A), JS (FIG. 9B) and MB (FIG. 9C) for binding to HuCOL-1 immobilized on a sensor chip. Percent binding of the sera to HuCOL-1 was calculated from the sensograms and plotted as a function of the concentration of the competitor.

FIG. 10 is a graph that represents sera reactivity, by SPR, of mCOL-1 and the engineered Abs derived from it. Increasing concentrations of mCOL-1 (■), HuCOL-1 (▲) and ^{24,25,27}L/^{61}H (●) were used to compete with the anti-V region Abs to COL-1 present in serum from patient MB for binding to mCOL-1 immobilized on a sensor chip. Percent binding of the sera to mCOL-1 was calculated from the sensograms and plotted as a function of the concentration of the competitor.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is

shown, but the complementary strand is understood as included by any reference to the displayed strand. In the accompanying sequence listing:

SEQ ID NO: 1 is the nucleic acid sequence of a murine COL-1 variable heavy chain 5' primer.

SEQ ID NO: 2 is the nucleic acid sequence of a murine COL-1 variable heavy chain 3' primer.

SEQ ID NO: 3 is the nucleic acid sequence of a murine COL-1 variable light chain 5' primer.

SEQ ID NO: 4 is the nucleic acid sequence of a murine COL-1 variable light chain 3' primer.

SEQ ID NO: 5 is the nucleic acid sequence of a humanized COL-1 variable heavy chain 5' primer.

SEQ ID NO: 6 is the nucleic acid sequence of a humanized COL-1 variable heavy chain 3' primer.

SEQ ID NO: 7 is the nucleic acid sequence of a humanized COL-1 variable light chain 5' primer.

SEQ ID NO: 8 is the nucleic acid sequence of a humanized COL-1 variable light chain 3' primer.

SEQ ID NO: 9 is the nucleic acid sequence of a humanized COL-1 variant variable heavy chain 3' primer.

SEQ ID NO: 10 is the nucleic acid sequence of a humanized COL-1 variant variable light chain 3' primer.

SEQ ID NO: 11 is the nucleotide sequence of the gene encoding the humanized V_L region of HuCOL-1 and its leader peptide.

SEQ ID NO: 12 is the nucleotide sequence of the gene encoding the humanized V_H region of HuCOL-1 and its leader peptide.

SEQ ID NO: 13 is the nucleotide sequence of the mCOL-1 V_L region.

SEQ ID NO: 14 is the nucleotide sequence of the mCOL-1 V_H region.

SEQ ID NO: 15 is amino acid sequence of the mCOL-1 V_L region.

SEQ ID NO: 16 is amino acid sequence of the mCOL-1 V_H region.

SEQ ID NO: 17 is amino acid sequence of the V_L region of the human antibody VJI'CL (GenBank Accession Number Z00022).

SEQ ID NO: 18 is the amino acid sequence of the V_L region of HuCOL-1 derived from mCOL-1 and VJI'CL.

SEQ ID NO: 19 is the amino acid sequence of the V_L region of HuCOL-1 variant ^{24,25,27}L (GenBank Accession Number PTA-4643).

SEQ ID NO: 20 is amino acid sequence of the V_H region of the human antibody MO30 (GenBank Accession Number A32483).

SEQ ID NO: 21 is the amino acid sequence of the V_H region of HuCOL-1 derived from mCOL-1 and MO30 (GenBank Accession Number PTA-4661).

SEQ ID NO: 22 is the amino acid sequence of the V_H region of HuCOL-1 variant ^{61}H (GenBank Accession Number PTA-4642).

SEQ ID NO: 23 is the amino acid sequence of CDR1 in the V_L region of murine COL-1.

SEQ ID NO: 24 is the amino acid sequence of CDR1 in the V_L region of human Ab VJI'CL.

SEQ ID NO: 25 is the amino acid sequence of CDR1 in the V_L region of HuCOL-1 derived from mCOL-1 and VJI'CL.

SEQ ID NO: 26 is the amino acid sequence of CDR1 in the V_L region of the HuCOL-1 variant ^{24,25,27}L.

SEQ ID NO: 27 is the amino acid sequence of CDR2 in the V_H region of mCOL-1.

SEQ ID NO: 28 is the amino acid sequence of CDR2 in the V_H region of human antibody MO30.

SEQ ID NO: 29 is the amino acid sequence of CDR2 in the V_H region of HuCOL-1 derived from mCOL-1 and MO30.

SEQ ID NO: 30 is the amino acid sequence of CDR2 in the V_H region of HuCOL-1 variant ^{61}H.

DETAILED DESCRIPTION

I. Abbreviations

Ab	antibody
Abs	antibodies
bp	base pair
C	constant
cCOL-1	chimeric COL-1
CEA	carcinoembryonic antigen
CH	constant heavy
CL	constant light
CDR	complementarity determining region
Fab	fragment antigen binding
$F(ab')_2$	Fab with additional amino acids, including cysteines necessary for disulfide bonds
FACS	fluorescence activated cell sort
FR	framework region
Fv	fragment variable
H	heavy
HAMA	human anti-murine antibody
HuCOL-1	humanized COL-1
HuIgG	human immunoglobulin G
Ig	immunoglobulin
Ka	relative binding affinity constant
L	light
mAb	monoclonal antibody
mCOL-1	murine COL-1
PCR	polymerase chain reaction
RIA	radioimmunoassay

scFv	single chain Fv
SDR	specificity determining residue
SPR	surface plasmon resonance
V	variable
VH	variable heavy
VL	variable light

II. Terms

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the invention, the following explanations of specific terms are provided:

Animal: Living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals. Similarly, the term "subject" includes both human and veterinary subjects.

Antibody: Immunoglobulin (Ig) molecules and immunologically active portions of Ig molecules, i.e., molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen. In one embodiment the antigen is CEA. Monoclonal and humanized immunoglobulins are encompassed by the disclosure. In one embodiment, a murine monoclonal antibody that recognizes CEA is COL-1. In another embodiment, a humanized COL-1 antibody is HuCOL-1 (ATCC Accession Number PTA-4661). In other embodiments, variant HuCOL-1 antibodies are HuCOL-

1^{61}H (ATCC Accession Number PTA-4642), HuCOL-$1^{24,25,27}$L (ATCC Accession Number PTA-4643), or HuCOL-$1^{24,25,27}$L/^{61}H (ATCC Accession Number PTA-4644). The disclosure also includes synthetic and genetically engineered variants of these immunoglobulins.

A naturally occurring antibody (e.g., IgG) includes four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. However, it has been shown that the antigen-binding function of an antibody can be performed by fragments of a naturally occurring antibody. Thus, these antigen-binding fragments are also intended to be designated by the term "antibody." Examples of binding fragments encompassed within the term antibody include (i) an Fab fragment consisting of the V_L, V_H, C_L and C_H1 domains; (ii) an Fd fragment consisting of the V_H and C_H1 domains; (iii) an Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (iv) a dAb fragment (Ward *et al.*, *Nature* 341:544, 1989) which consists of a V_H domain; and (v) an F(ab')$_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region. Furthermore, although the two domains of the Fv fragment are coded for by separate genes, a synthetic linker can be made that enables them to be made as a single protein chain (known as single chain Fv (scFv); Bird *et al. Science* 242:423, 1988; and Huston *et al. Proc. Natl. Acad. Sci.* 85:5879, 1988) by recombinant methods. Such single chain antibodies, as well as dsFv, a disulfide stabilized Fv (Bera *et al. J. Mol. Biol.* 281:475, 1998), and dimeric Fvs (diabodies), that are generated by pairing different polypeptide chains (Holliger *et al. Proc. Natl. Acad. Sci.* 90:6444, 1993), are also included.

In one embodiment, antibody fragments for use in this disclosure are those which are capable of cross-linking their target antigen, e.g., bivalent fragments such as F(ab')$_2$ fragments. Alternatively, an antibody fragment which does not itself cross-link its target antigen (e.g., a Fab fragment) can be used in conjunction with a secondary antibody which serves to cross-link the antibody fragment, thereby cross-linking the target antigen. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described for whole antibodies.

An antibody is further intended to include humanized monoclonal molecules that specifically bind the target antigen.

"Specifically binds" refers to the ability of individual antibodies to specifically immunoreact with an antigen. This binding is a non-random binding reaction between an antibody molecule and the antigen. In one embodiment, the antigen is CEA. Binding specificity is typically determined from the reference point of the ability of the antibody to differentially bind the antigen of interest and an unrelated antigen, and therefore distinguish between two different antigens, particularly where the two antigens have unique epitopes. An antibody that specifically binds to a particular epitope is referred to as a "specific antibody".

A variety of methods for linking effector molecules to antibodies are well known in the art. Detectable labels useful for such purposes are also well known in the art, and include radioactive isotopes such as ^{32}P, fluorophores, chemiluminescent agents, and enzymes. Also encompassed in the disclosure are the chemical or biochemical modifications that incorporate toxins in the antibody. In one embodiment, the toxin is chemically conjugated to the antibody. In another embodiment, a fusion protein is genetically engineered to include the antibody and the toxin. Specific, non-limiting examples of toxins are radioactive isotopes, chemotherapeutic agents, bacterial toxins, viral toxins, or venom proteins. The disclosure also includes chemical or genetically engineered modifications that link a cytokine to an antibody (such as by a covalent linkage). Specific, non-limiting examples of cytokines are interleukin (IL)-2, IL-4, IL-10, tumor necrosis factor (TNF)-alpha and interferon (IFN)-gamma..

Antigen: Any molecule that can bind specifically with an antibody.. An antigen is also a substance that antagonizes or stimulates the immune system to produce antibodies. Antigens are often foreign substances such as allergens, bacteria or viruses that invade the body. One specific, non-limiting example of an antigen is CEA.

Carcinoembryonic antigen (CEA): A member of the immunoglobulin superfamily that includes normal fecal antigen, non-specific cross-reacting antigen, meconium antigen, and biliary glycoprotein. CEA is composed of seven domains linked to the cell membrane through a glycosylphosphatidylinositol anchor and has a

molecular weight of 180 kDa (GenBank Accession Number A36319, herein incorporated by reference). CEA is normally expressed in a variety of glandular epithelial tissues, where it appears to be localized to the apical surface of the cells, although it is also expressed in numerous carcinomas including gastrointestinal, colorectal, breast, ovarian and lung carcinomas (Robbins *et al., Int'l J. Cancer*, 53:892-897, 1993; Greiner *et al., J. Clin. Oncol.*, 10:735-746, 1992; Ohuchi *et al., Cancer Res.* 47:3565-5780, 1985; Muraro *et al., Cancer Res.*, 45:57695780, 1985). CEA is an especially well characterized human tumor antigen and is widely used for the diagnosis of human colon cancer. Monoclonal antibodies, designated COL-1 through COL-15, have been generated to detect various epitopes on CEA (Muraro *et al., Cancer Res.*, 45:5769-5780, 1985, herein incorporated by reference), and in using these antibodies the differential expression of CEA has been determined (Muraro *et al., Cancer Res.*, 45:5769-5780, 1985; Ohuchi *et al., Cancer Res.* 47:3565-3571, 1987; Wilkinson *et al., Proc. Natl. Acad. Sci.* 98:10256, 2001). Of these monoclonal antibodies, COL-1 is of clinical importance because it has a high affinity for CEA.

cDNA (complementary DNA): A piece of DNA lacking internal, non-coding segments (introns) and regulatory sequences that determine transcription. cDNA is synthesized in the laboratory by reverse transcription from messenger RNA extracted from cells.

Chimeric antibody: An antibody which includes sequences derived from two different antibodies, which typically are of different species. Most typically, chimeric antibodies include human and murine antibody domains, generally human constant and murine variable regions.

Complementarity Determining Region (CDR): Amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native Ig binding site. The light and heavy chains of an Ig each have three CDRs, designated L-CDR1, L-CDR2, L-CDR3 and H-CDR1, H-CDR2, H-CDR3, respectively. By definition, the CDRs of the light chain are bounded by the residues at positions 24 and 34 (L-CDR1), 50 and 56 (L-CDR2), 89 and 97 (L-CDR3); the CDRs of the heavy chain are bounded by the residues at positions 31 and 35b (H-CDR1), 50 and 65 (H-CDR2),

95 and 102 (H-CDR3), using the numbering convention delineated by Kabat *et al.*, (1991) *Sequences of Proteins of Immunological Interest*, 5th Edition, Department of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda (NIH Publication No. 91-3242).

Constant Region: The portion of the antibody molecule which confers effector functions. In the present disclosure, the variant antibodies include constant regions derived from human immunoglobulins. The heavy chain constant region can be selected from any of five isotypes: alpha, delta, epsilon, gamma or mu. Heavy chains of various subclasses (such as the IgG subclass of heavy chains) are responsible for different effector functions. Thus, by choosing the desired heavy chain constant region, humanized antibodies with the desired effector function can be produced. The light chain constant region can be of the kappa or lambda type.

Cytotoxin: An agent that is toxic for cells. Examples of cytotoxins include radioactive isotopes, chemotherapeutic drugs, bacterial toxins, viral toxins, and proteins contained in venom (e.g. insect, spider, reptile, or amphibian venom). A cytokine, such as interleukin-2 or interferon, can also be a cytotoxin.

DNA: Deoxyribonucleic acid. DNA is a long chain polymer which constitutes the genetic material of most living organisms (some viruses have genes composed of ribonucleic acid (RNA)). The repeating units in DNA polymers are four different nucleotides, each of which contains one of the four bases, adenine, guanine, cytosine and thymine bound to a deoxyribose sugar to which a phosphate group is attached. Triplets of nucleotides (referred to as codons) code for each amino acid in a polypeptide. The term codon is also used for the corresponding (and complementary) sequence of three nucleotides in the mRNA that is transcribed from the DNA.

Encode: A polynucleotide is said to "encode" a polypeptide if, in its native state or when manipulated by methods well known to those skilled in the art, it can be transcribed and/or translated to produce the mRNA for the polypeptide and/or a fragment thereof. The anti-sense strand is the complement of such a nucleic acid, and the encoding sequence can be deduced therefrom.

Epitope: A site on an antigen recognized by an antibody, as determined by the specificity of the antibody amino acid sequence. Epitopes are also called antigenic determinants.

Framework Region: Amino acid sequences interposed between CDRs include variable light and variable heavy framework regions. The framework regions serve to hold the CDRs in an appropriate orientation for antigen binding.

HAMA (Human anti-murine antibody) response: An immune response in a human subject to the variable and constant regions of a murine antibody that has been administered to the patient. Repeated antibody administration may lead to an increased rate of clearance of the antibody from the patient's serum and may also elicit allergic reactions in the patient.

Humanized antibody: A human antibody genetically engineered to include mouse hypervariable regions. In one embodiment, the DNA encoding hypervariable loops of mouse monoclonal antibodies or variable regions selected in phage display libraries is inserted into the framework regions of human Ig genes. Antibodies can be "customized" to have a desired binding affinity or to be minimally immunogenic in the humans treated with them.

Humanized COL-1 antibodies: COL-1 antibodies humanized by grafting mCOL-1 (murine COL-1) CDRs onto the frameworks of the relevant human antibodies. The murine CDRs in the resultant humanized COL-1 (HuCOL-1) could evoke an anti-idiotypic response when administered in human subjects. COL-1 can be humanized by grafting only a subset of the COL-1 CDR residues, for example those that are important for antigen binding, onto the variable light and variable heavy framework regions of, for example, VJI'CL and MO30 human antibodies. In one embodiment of an HuCOL-1 antibody, COL-1 CDR residues that are not involved in antigen binding (non-ligand contact residues) are substituted with the corresponding residues of a human antibody such as those from, for example, VJI'CL or MO30. One embodiment of a humanized COL-1 monoclonal antibody is HuCOL-1 (ATCC Accession Number PTA-4661). In another embodiment, a humanized COL-1 antibody is HuCOL-1^{24,25,27}L (ATCC Accession Number PTA-4643). In other embodiments, a humanized COL-1 antibody is

HuCOL-1^{61}H (ATCC Accession Number PTA-4642) and HuCOL-1^{24,25,27}L/^{61}H (ATCC Accession Number PTA-4644). HuCOL-1 is referred to herein as the parent HuCOL-1 antibody and both mCOL-1 and HuCOL-1 are referred to herein as parental antibodies. HuCOL-1^{61}H, HuCOL-1^{24,25,27}L, and HuCOL-1^{24,25,27}L/^{61}H are referred to herein as variant HuCOL-1 antibodies.

IC_{50} value: The concentration of a competitor antibody (for example, concentration of a variant HuCOL-1) required for half-maximal (50%) inhibition of binding of sera to another antibody (for example, mCOL-1 or the parent HuCOL-1). A higher IC_{50} value for a particular antibody indicates a decreased reactivity of that antibody to the serum, suggesting that the antibody with the higher IC_{50} value has reduced immunogenicity in a subject. In one embodiment, a variant HuCOL-1 antibody has an IC_{50} value that is greater than that of the parent HuCOL-1 antibody, suggesting that the variant HuCOL-1 antibody has a decreased immunogenicity in a subject compared to the parent HuCOL-1 antibody.

Idiotype: the property of a group of antibodies or T cell receptors defined by their sharing a particular idiotope (an antigenic determinant on the variable region); *i.e.*, antibodies that share a particular idiotope belong to the same idiotype. "Idiotype" may be used to describe the collection of idiotopes expressed by an Ig molecule. An "anti-idiotype" antibody may be prepared to a monoclonal antibody by methods known to those of skill in the art and may be used to prepare pharmaceutical compositions.

Immune cell: Any cell involved in a host defense mechanism. These can include, for example, T cells, B cells, natural killer cells, neutrophils, mast cells, macrophages, antigen-presenting cells, basophils, eosinophils, and neutrophils.

Immune response: A response of a cell of the immune system, such as a neutrophil, a B cell, or a T cell, to a stimulus. In one embodiment, the response is specific for a particular antigen (an "antigen-specific response"). In another embodiment, the response is against an antibody, such as HAMA response, including an anti-variable region response.

Immunoconjugate: A covalent linkage of an effector molecule to an antibody. The effector molecule can be a toxin or a detectable label. Specific, non-limiting

examples of toxins include, but are not limited to, abrin, ricin, *Pseudomonàs* exotoxin (such as PE35, PE37, PE38, and PE40), diphtheria toxin, anthrax toxin, botulinum toxin, or modified toxins thereof. For example, *Pseudomonas* exotoxin and diphtheria toxin are highly toxic compounds that typically bring about death through liver toxicity. *Pseudomonas* exotoxin and diphtheria toxin, however, can be modified into a form for use as an immunotoxin by removing the native targeting component of the toxin (*e.g.*, domain Ia of *Pseudomonas* exotoxin and the B chain of diphtheria toxin) and replacing it with a different targeting moiety, such as an antibody.

Other toxic agents, that directly or indirectly inhibit cell growth or kill cells, include chemotherapeutic drugs, cytokines, for example interleukin-2 or interferon, radioactive isotopes, viral toxins, or proteins contained within, for example, insect, spider, reptile, or amphibian venom. Specific, non-limiting examples of detectable labels include, but are not limited to, radioactive isotopes, enzyme substrates, co-factors, ligands, chemiluminescent agents, fluorescent agents, haptens, or enzymes. A "chimeric molecule" is a targeting moiety, such as a ligand or an antibody, conjugated (attached or coupled) to an effector molecule.

The term "conjugated" or "linked" refers to making two polypeptides into one contiguous polypeptide molecule. In one embodiment, an antibody is joined to an effector molecule. In another embodiment, an antibody joined to an effector molecule is further joined to a lipid or other molecule to a protein or peptide to increase its half-life in the antibody. Therapeutic, diagnostic or detection moieties can be linked to an antibody using any number of means known to those of skill in the art. Both covalent and noncovalent linkage means may be used. The procedure for linking an effector molecule to an antibody varies according to the chemical structure of the effector. Polypeptides typically contain a variety of functional groups; *e.g.*, carboxyl (COOH), amino ($-NH_2$) or sulfhydryl (-SH) groups, which are available for reaction with a suitable functional group on an antibody to result in the linkage of the effector molecule. Alternatively, the antibody is derivatized to expose or link additional reactive functional groups. The derivatization may involve linkage of any of a number of linker molecules such as those available from Pierce Chemical Company, Rockford Illinois.

The linker can be any molecule used to join the antibody to the effector molecule. The linker is capable of forming covalent bonds to both the antibody and to the effector molecule. Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers. Where the antibody and the effector molecule are polypeptides, the linkers may be joined to the constituent amino acids through their side groups (*e.g.*, through a disulfide linkage to cysteine) or to the alpha carbon amino and carboxyl groups of the terminal amino acids.

In some circumstances, it is desirable to free the effector molecule from the antibody when the immunoconjugate has reached its target site. Therefore, in these circumstances, immunoconjugates will comprise linkages that are cleavable in the vicinity of the target site. Cleavage of the linker to release the effector molecule from the antibody may be prompted by enzymatic activity or conditions to which the immunoconjugate is subjected either inside the target cell or in the vicinity of the target site. When the target site is a tumor, a linker which is cleavable under conditions present at the tumor site (*e.g.* when exposed to tumor-associated enzymes or acidic pH) may be used.

In view of the large number of methods that have been reported for linking a variety of radiodiagnostic compounds, radiotherapeutic compounds, label (e.g. enzymes or fluorescent molecules) drugs, toxins, and other agents to antibodies one skilled in the art will be able to determine a suitable method for linking a given agent to an antibody.

Immunogenicity: A measure of the ability of a targeting protein or therapeutic moiety to elicit an immune response (humoral or cellular) when administered to a subject.

Immunoreactivity: A measure of the ability of an Ig to recognize and bind to a specific antigen.

Isolated: An biological component (such as a nucleic acid, peptide or protein) that has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, *i.e.*, other chromosomal and extrachromosomal DNA and RNA, and proteins.

Nucleic acids, peptides and proteins that have been "isolated" thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids, peptides and proteins prepared by recombinant DNA expression in a host cell as well as chemically synthesized nucleic acids.

Label: A detectable compound or composition that is conjugated directly or indirectly to another molecule to facilitate detection of that molecule. Specific, non-limiting examples of labels include fluorescent tags, chemiluminescent tags, haptens, enzymatic linkages, and radioactive isotopes.

Ligand contact residue: A residue within a CDR that is involved in contact with a ligand or antigen. A ligand contact residue is also known as a specificity determining residue (SDR). A non-ligand contact residue is a residue in a CDR that does not contact a ligand. A non-ligand contact residue can also be a framework residue.

Lymphocytes: A type of white blood cell that is involved in the immune defenses of the body. There are two main types of lymphocytes: B-cells and T-cells.

Mammal: This term includes both human and non-human mammals. Similarly, the term "subject" includes both human and veterinary subjects.

Minimally immunogenic: An antibody that generates a reduced, for example low, immune response when administered to a subject, such as a human subject. In one embodiment, a minimally immunogenic antibody is an antibody that is administered to a subject without eliciting a HAMA response. In one embodiment, immunogenicity is measured in a competitive binding assay. In one specific, non-limiting example, immunogenicity is the ability of a variant HuCOL-1 antibody to prevent a mCOL-1 or parental HuCOL-1 antibody from binding to COL-1 anti-idiotypic antibodies in a patient's serum. If a variant HuCOL-1 antibody competes with an equal molar amount of the parental HuCOL-1 antibody (*i.e.* elicits greater than about 50% inhibition of parental HuCOL-1 binding to anti-idiotypic antibodies in a patient's serum) then the variant HuCOL-1 antibody is immunogenic. If a variant HuCOL-1 antibody competes poorly with an equal molar or less amount of the parent HuCOL-1 antibody (*i.e.* elicits about 50% or less inhibition of parental HuCOL-1 binding to anti-idiotypic antibodies

in a patient's serum) then the variant HuCOL-1 antibody is minimally immunogenic. IC_{50} is the concentration of the competitor antibody (for example, concentration of a variant HuCOL-1) required for half-maximal (50%) inhibition of binding of sera to mCOL-1, or the parent HuCOL-1.

Monoclonal antibody: An antibody produced by a single clone of B-lymphocytes. Monoclonal antibodies are produced by methods known to those of skill in the art, for instance by making hybrid antibody-forming cells from a fusion of myeloma cells with immune spleen cells.

Nucleic acid: A deoxyribonucleotide or ribonucleotide polymer in either single or double stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in a manner similar to naturally occurring nucleotides.

Oligonucleotide: A linear single-stranded polynucleotide sequence of up to about 200 nucleotide bases in length, for example a polymer of deoxyribonucleotides or ribonucleotides which is at least 6 nucleotides, for example at least 15, 50, 100 or even 200 nucleotides long.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

Phage display: A technique wherein DNA sequences are amplified and cloned into filamentous phage vector to create a library of fusion phages ("phage library") in which the phages display on their surface the proteins encoded by the foreign DNA. In one embodiment, a phage library is produced that expresses HuCOL-1 variant immunoglobulins. From the rescued phages, the individual phage clones are selected through interaction of the displayed protein with a ligand, and the specific phage is amplified by infection of bacteria. Antigen specific immunoglobulins can then be

expressed and characterized for their antigen binding and sera reactivity (potential immunogenicity).

Pharmaceutical agent: A chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject or a cell. "Incubating" includes a sufficient amount of time for a drug to interact with a cell. "Contacting" includes incubating a drug in solid or in liquid form with a cell.

A "therapeutically effective amount" is a quantity of a specific substance sufficient to achieve a desired effect in a subject being treated. For instance, this can be the amount necessary to inhibit or suppress growth of a tumor or to decrease a sign or symptom of the tumor in the subject. In one embodiment, a therapeutically effective amount is the amount necessary to eliminate a tumor. When administered to a subject, a dosage will generally be used that will achieve target tissue concentrations (for example, in tumors) that has been shown to achieve a desired *in vitro* effect.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers useful in this disclosure are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of humanized COL-1 monoclonal antibodies disclosed herein.

In general, the nature of the carrier will depend on the particular mode of administration employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (e.g., powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Polynucleotide: A single-stranded linear nucleotide sequence, including sequences of greater than 100 nucleotide bases in length.

Polypeptide: A polymer in which the monomers are amino acid residues that are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used, the L-isomers being preferred in nature. The term polypeptide or protein as used herein encompasses any amino acid sequence and includes, but may not be limited to, modified sequences such as glycoproteins. The term polypeptide is specifically intended to cover naturally occurring proteins, as well as those that are recombinantly or synthetically produced.

Substantially purified polypeptide as used herein refers to a polypeptide that is substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. In one embodiment, the polypeptide is at least 50%, for example at least 80% free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. In another embodiment, the polypeptide is at least 90% free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. In yet another embodiment, the polypeptide is at least 95% free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated.

Conservative amino acid substitution tables providing functionally similar amino acids are well known to one of ordinary skill in the art. The following six groups are examples of amino acids that are considered to be conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

A non-conservative amino acid substitution can result from changes in: (a) the structure of the amino acid backbone in the area of the substitution; (b) the charge or hydrophobicity of the amino acid; or (c) the bulk of an amino acid side chain.

Substitutions generally expected to produce the greatest changes in protein properties are those in which: (a) a hydrophilic residue is substituted for (or by) a hydrophobic residue; (b) a proline is substituted for (or by) any other residue; (c) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine; or (d) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl.

Variant amino acid sequences may, for example, be 80, 90 or even 95 or 98% identical to the native amino acid sequence. Programs and algorithms for determining percentage identity can be found at the NCBI website.

Preventing or treating a disease: Preventing a disease refers to inhibiting completely or in part the development or progression of a disease, for example in a person who is known to have a predisposition to a disease. An example of a person with a known predisposition is someone with a history of cancer in the family, or who has been exposed to factors that predispose the subject to the development of a tumor. Treating a disease refers to a therapeutic intervention that inhibits, or suppressed the growth of a tumor, eliminates a tumor, ameliorates at least one sign or symptom of a disease or pathological condition, or interferes with a pathophysiological process, after the disease or pathological condition has begun to develop.

Protein: A biological molecule encoded by a gene and comprised of amino acids.

Recombinant: A recombinant nucleic acid is one that has a sequence that is not naturally occurring or was made artificially. Artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. Similarly, a recombinant protein is one encoded by a recombinant nucleic acid molecule.

Relative binding affinity constant (Ka): Affinity of an antibody for an antigen can be expressed relative to the binding affinity of another antibody to the same antigen. In several embodiments, the relative affinity constant of a variant HuCOL-1 antibody is

less than, similar to, or greater than, that of a murine COL-1 or a parent HuCOL-1 antibody. In one embodiment, affinity is calculated by a modification of the Scatchard method described by Frankel *et al. Mol. Immunol.*, 16:101, 1979. One of skill in the art can readily identify a statistical test that determines a statistically significant result for example, the Student's t-test, the Wilcoxon two sample test, or the Median test. In one embodiment, a variant HuCOL-1 antibody retains CEA binding affinity if the variant HuCOL-1 antibody binds CEA and has a relative binding affinity constant at least about 1.0×10^{-8} M. In other embodiments, a variant HuCOL-1 antibody retains CEA binding affinity if the relative binding affinity constant is at least about 1.2×10^{-8}, about 1.5×10^{-8}, about 2.0×10^{-8}, about 2.5×10^{-8}, about 2.8×10^{-8}, about 3.0×10^{-8}, about 3.2×10^{-8}, about 3.5×10^{-8}, about 4.0×10^{-8}, about 4.5×10^{-8}, or about 5.0×10^{-8} M.

In another embodiment, a binding affinity is measured by an antigen/antibody dissociation rate. In yet another embodiment, a binding affinity is measured by a competition radioimmunoassay. In a further embodiment, a binding affinity is measured by flow cytometry as the number of gated cells labeled with HuCOL-1 antibody.

Subject: Living multi-cellular vertebrate organisms, a category that includes both human and non-human mammals.

Therapeutically effective amount: A quantity of a specific substance sufficient to achieve a desired effect in a subject being treated. For instance, this can be the amount necessary to inhibit or suppress growth of a tumor. In one embodiment, a therapeutically effective amount is the amount necessary to eliminate a tumor. When administered to a subject, a dosage will generally be used that will achieve target tissue concentrations (for example, in tumors) that has been shown to achieve a desired *in vitro* effect.

Treatment: Refers to both prophylactic inhibition of initial infection or disease, and therapeutic interventions to alter the natural course of an untreated infection or disease process, such as a tumor growth.

Tumor: A neoplasm that may be either malignant or non-malignant. Tumors of the same tissue type are primary tumors originating in a particular organ (such as

breast, gastrointestinal, prostate, ovarian, bladder or lung). Tumors of the same tissue type may be divided into tumor of different sub-types (a classic example being bronchogenic carcinomas (lung tumors) which can be an adenocarcinoma, small cell, squamous cell, or large cell tumor). Breast cancers can be divided histologically into scirrhous, infiltrative, papillary, ductal, medullary and lobular. In one embodiment, cells in a tumor express CEA.

Variable region (also variable domain or V domain): The regions of both the light-chain and the heavy-chain on an Ig that contain antigen-binding sites. The regions are composed of polypeptide chains containing four relatively invariant "framework regions" (FRs) and three highly variant "hypervariable regions" (HVs). Because the HVs constitute the binding site for antigen(s) and determine specificity by forming a surface complementarity to the antigen, they are more commonly termed the "complementarity-determining regions," or CDRs, and are denoted CDR1, CDR2, and CDR3. Because both of the CDRs from the heavy- and light-chain domains contribute to the antigen-binding site, it is the three-dimensional combination of the heavy and the light chain that determines the final antigen specificity.

Within the heavy- and light-chain, the framework regions surround the CDRs. Proceeding from the N-terminus of a heavy or light chain, the order of regions is: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. As used herein, the term "variable region" is intended to encompass a complete set of four framework regions and three complementarity-determining regions. Thus, a sequence encoding a "variable region" would provide the sequence of a complete set of four framework regions and three complementarity-determining regions.

Variant HuCOL-1: A humanized COL-1 antibody that has at least one amino acid substitution in a murine CDR region.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to

include "and" unless the context clearly indicates otherwise. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Humanized COL-1 antibodies

Disclosed herein are humanized COL-1 (HuCOL-1) monoclonal antibodies that have an amino acid substitution in the heavy chain complementarity determining region (HCDR) 2 of the COL-1 antibody. HuCOL-1 antibodies with an amino acid substitution are also referred to herein as variant HuCOL-1 antibodies. In one embodiment, the variant HuCOL-1 antibody has an amino acid substitution of a non-ligand contact residue in HCDR2. In other embodiments, the variant HuCOL-1 antibody has an amino acid substitution of a non-ligand contact residue at position 59, 60, 61, 62, 63, 64, or 65 of HCDR2 (FIG. 3). In one example, the variant HuCOL-1 antibody has an amino acid substitution at position 61 of HCDR2, such as a proline to glutamine substitution (see HuCOL-1^{61}H in Table 1).

Table 1. Affinity constants of HuCOL-1 and its variants

MAb Designation	Light Chain Variable Domain	Heavy Chain Variable Domain	Manipulated CDR	Positions Substituted[a]	Ka ($x10^8 M^{-1}$)
HuCOL-1	Humanized	Humanized			2.82
^{24,25,27}L	Variant	Humanized	LCDR1	24, 25, 27	1.20
^{61}H	Humanized	Variant	HCDR2	61	2.64
^{24,25,27}L/^{61}H	Variant	Variant	LCDR1/HCDR2	24,25,27/61	1.03

[a] Numbering convention of Kabat *et al.* (Sequence of proteins of immunological interests, 5th Ed. U.S. Department of Health and Human Services, National Institutes of Health, Bethesda, MD (NIH Publication no. 91-3242), 1991)

In one embodiment, the variant HuCOL-1 antibody has no more than one amino acid substitution in HCDR2. However, the variant HuCOL-1 antibody can include no more than two, no more than three, no more than four, or no more than seven amino acid substitutions in HCDR2. In some embodiments, the variant HuCOL-1 antibody has an amino acid substitution at position 61 of HCDR2 and an additional amino acid substitution at 59, 60, 62, 63, 64, or 65 of HCDR2. In another embodiment, the variant HuCOL-1 antibody has an amino acid substitution at position 61 of HCDR2 and an additional amino acid substitution of a ligand contact residue in HCDR2.

The variant HuCOL-1 antibody can have an amino acid substitution in the light chain complementarity determining region (LCDR) 1 of the COL-1 antibody. In one embodiment, the variant HuCOL-1 antibody has an amino acid substitution of a non-ligand contact residue in LCDR1. In other embodiments, the HuCOL-1 antibody has an amino acid substitution of a non-ligand contact residue at position 24, 25, 26, 27, 27a, 27b, or 27c of LCDR1 (FIG. 3). In one embodiment, the variant HuCOL-1 antibody has no more than one amino acid substitution in LCDR1. However, in other examples the variant HuCOL-1 antibody includes no more than two, no more than three, no more

than four, or no more than seven amino acid substitutions in LCDR1. In one embodiment, the variant HuCOL-1 antibody has an amino acid substitution at position 24, 25 and 27 of LCDR1. In one specific, non-limiting example, the variant HuCOL-1 antibody has an arginine to lysine substitution at position 24 of LCDR1. In another specific, non-limiting example, the variant HuCOL-1 antibody has an alanine to serine substitution at position 25 of LCDR1. In yet another specific, non-limiting example, the variant HuCOL-1 antibody has a lysine to glutamine substitution at position 27 of LCDR1. In one specific, non-limiting example, the variant HuCOL-1 antibody has an arginine to lysine substitution at position 24, an alanine to serine substitution at position 25 of LCDR1, and a lysine to glutamine substitution at position 27 of LCDR1 (see HuCOL-$1^{24,25,27}$L in Table 1). In one embodiment, the variant HuCOL-1 antibody has an amino acid substitution at position 24, 25, and 27 of LCDR1 and an additional amino acid substitution of a ligand contact residue in LCDR1.

In some examples, a variant HuCOL-1 monoclonal antibody includes an amino acid substitution in HCDR2 and an additional amino acid substitution. For example, an additional substitution is an amino acid substitution in another HCDR or in an LCDR. One specific, non-limiting example of a variant HuCOL-1 monoclonal antibody that includes amino acid substitutions in more than one CDR is a HuCOL-1 monoclonal antibody with an amino acid substitution of a non-ligand contact residue in HCDR2 and an amino acid substitution of a non-ligand contact residue in LCDR1. In one specific, non-limiting example, the HuCOL-1 antibody has an amino acid substitution at position 61 of HCDR2 and at positions 24, 25, and 27 of LCDR1 (see HuCOL-$1^{24,25,27}$L/^{61}H in Table1). In one embodiment, the variant HuCOL-1 with an amino acid substitution in HCDR2 has an additional amino acid substitution of a ligand contact residue in another HCDR or in an LCDR. In another embodiment, the variant HuCOL-1 antibody has an additional amino acid substitution in a framework residue.

Immunogenicity of variant HuCOL-1 antibodies can be measured in a competitive binding assay as the ability of a variant HuCOL-1 antibody to prevent a murine COL-1 or the parent HuCOL-1 antibody from binding to anti-idiotypic antibodies in a human subject's serum. In one embodiment, a variant HuCOL-1

antibody with an amino acid substitution in HCDR2, such as HuCOL-1^{61}H, HuCOL-$1^{24,25,27}$L, or HuCOL-$1^{24,25,27}$L/^{61}H, are minimally immunogenic in a subject.

In one embodiment, at least about a two-fold higher molar concentration of the variant HuCOL-1 antibody, compared to the molar concentration of mCOL-1 or the parent HuCOL-1 antibody, is required to elicit 50% inhibition of mCOL-1 or parent HuCOL-1 antibody binding, respectively, to its cognate anti-idiotypic antibodies in a subject's sera. In other embodiments, at least about a three-fold, at least about a five-fold, at least about a ten-fold, at least about fifteen-fold, at least about a twenty five-fold, at least about a fifty-fold, at least about a seventy-fold, or at least about a one hundred-fold higher molar concentration of the variant HuCOL-1 antibody, compared to the molar concentration of the mCOL-1 or the parent HuCOL-1 antibody, is required to elicit 50% inhibition of mCOL-1 or parent HuCOL-1 antibody binding, respectively, to its cognate anti-idiotypic antibodies in a subject's serum. In one specific, non-limiting example, at least about a seventeen-fold higher molar concentration of the variant HuCOL-$1^{24,25,27}$L/^{61}H antibody, compared to the molar concentration of mCOL-1 monoclonal antibody, is required to elicit 50% inhibition of mCOL-1 antibody binding to its cognate anti-idiotypic antibodies in a subject's serum. In another specific, non-limiting example, at least about a five-fold higher molar concentration of the variant HuCOL-$1^{24,25,27}$L/^{61}H antibody, compared to the molar concentration of parent HuCOL-1 monoclonal antibody, is required to elicit 50% inhibition of HuCOL-1 antibody binding to its cognate anti-idiotypic antibodies in a subject's serum.

In one embodiment, HuCOL-1 has minimal immunogenicity, compared to the mCOL-1 antibody, since at least a 3-fold higher molar concentration of HuCOL-1 was required to attain 50% inhibition of mCOL-1 binding to patient serum. In another embodiment, HuCOL-1 suffered a partial loss in antigen-binding affinity (2.82×10^{-8} M) compared to the mCOL-1 antibody (5.17×10^{-8} M). In another embodiment, HuCOL-$1^{24,25,27}$L/^{61}H has minimal immunogenicity, compared to the mCOL-1 and HuCOL-1 antibodies, since at least 17-fold and 5.5-fold higher molar concentrations of HuCOL-$1^{24,25,27}$L/^{61}H were required to attain 50% inhibition of mCOL-1 and HuCOL-1 binding, respectively, to patient serum. In another embodiment, HuCOL-$1^{24,25,27}$L/^{61}H

suffered a partial loss in antigen binding affinity (1.03×10^{-8} M) compared to that of HuCOL-1 (2.64×10^{-8} M).

The variant HuCOL-1 antibodies disclosed herein retain CEA relative binding affinity. In one embodiment, a variant HuCOL-1 antibody retains CEA binding affinity if the relative binding affinity constant is at least about 1.0×10^{-8} M. In other embodiments, a variant HuCOL-1 antibody retains CEA binding affinity if the relative binding affinity constant is at least about 1.2×10^{-8}, about 1.5×10^{-8}, about 2.0×10^{-8}, about 2.5×10^{-8}, about 2.8×10^{-8}, about 3.0×10^{-8}, about 3.2×10^{-8}, about 3.5×10^{-8}, about 4.0×10^{-8}, about 4.5×10^{-8}, or about 5.0×10^{-8} M. In specific, non-limiting examples, HuCOL-$1^{24,25,27}$L, HuCOL-1^{61}H, and HuCOL-$1^{24,25,27}$L/^{61}H have relative binding affinity constants for CEA that are about 1.20×10^{-8} M, about 2.64×10^{-8} M, and about 1.03×10^{-8} M, respectively.

In one embodiment, the variant HuCOL-1 antibody retains CEA binding affinity if the variant HuCOL-1 antibody can inhibit the binding of mCOL-1 or HuCOL-1 to CEA. In one embodiment, the variant HuCOL-1 antibody retains CEA binding affinity, compared to the mCOL-1 or the parent HuCOL-1 antibody, if the variant has a similar antigen/antibody dissociation rate as that of the mCOL-1 or the parent HuCOL-1 antibody. In other embodiments, the variant HuCOL-1 antibody retains CEA binding affinity if a similar amount of the variant antibody is required for a 50% inhibition of the binding of ^{125}I-labeled HuCOL-1 to CEA, compared to the murine COL-1 antibody or the parent HuCOL-1 antibody. In a further embodiment, the variant HuCOL-1 antibody retains CEA binding affinity when cells expressing CEA are labeled with the variant HuCOL-1 antibody. For example, the number of CEA-expressing cells labeled with the variant HuCOl-1 antibody can be measured by flow cytometry. In specific, non-limiting examples the variant HuCOL-1 antibody retains CEA binding affinity when the number of CEA-expressing cells labeled by a variant HuCOL-1 antibody is similar to the number of CEA-expressing cells labeled by the murine COL-1 antibody or the parent HuCOL-1 antibody.

In one embodiment, HuCOL-1 has a relative binding affinity constant for CEA (2.82×10^{-8} M) that is 1.8-fold less than that of mCOL-1 (5.17×10^{-8} M) In other

embodiments, HuCOL-$1^{24,25,27}$L, HuCOL-1^{61}H, and HuCOL-$1^{24,25,27}$L/^{61}H have relative binding affinity constants for CEA (1.20×10^{-8} M, 2.64×10^{-8} M, and 1.03×10^{-8} M, respectively) that are 4.3-, 1.9-, and 5.0-fold less than that of mCOL-1. In yet other embodiments, HuCOL-$1^{24,25,27}$L and HuCOL-$1^{24,25,27}$L/^{61}H have relative binding affinity constants for CEA (1.20×10^{-8} M and 1.03×10^{-8} M, respectively) that are 2-fold less than that of HuCOL-1. In another embodiment, HuCOL-1^{61}H has a relative binding affinity constant (2.64×10^{-8} M) that is similar to that of HuCOL-1 (2.82×10^{-8} M).

In one embodiment, the variant HuCOL-1 antibody has an increase in CEA binding affinity, compared to the murine COL-1 antibody, if the variant has a lower antigen/antibody dissociation rate compared to that of the murine COL-1 or the parent HuCOL-1 antibody. In other embodiments, the variant HuCOL-1 antibody has an increase in CEA binding affinity if less of the variant antibody is required for a 50% inhibition of the binding of ^{125}I-labeled HuCOL-1 to CEA, compared to the murine COL-1 antibody or the parent HuCOL-1 antibody. In further embodiments, the variant HuCOL-1 antibody has an increase in CEA binding affinity when the number of CEA-expressing cells labeled with variant HuCOL-1 antibody is significantly greater than the number of CEA-expressing cells labeled by the murine COL-1 antibody or the parent HuCOL-1 antibody.

In one embodiment, the variant HuCOL-1 antibody has a CH2 domain deletion (Slavin-Chiorini *et al., Int. J. Cancer*, 53:97-103, 1993; Slavin-Chiorini *et al., Cancer Research*, 55:5957s-5967s, 1995; Slavin-Chiorini *et al., Cancer Biother. Radiopharm.*, 12:305-316, 1997, incorporated herein by reference). The generation and characterization of CH2 domain deleted antibodies is described in Mueller *et al., Proc. Natl. Acad. Sci. USA.*, 87:5702-5705, 1990. In one specific embodiment, a variant HuCOL-1 antibody with a CH2 domain deletion is cleared more quickly from the plasma compared to the murine COL-1 monoclonal antibody or the parent HuCOL-1 antibody. In other specific embodiments, a variant HuCOL-1 antibody with a CH2 domain deletion has reduced immunogenicity compared to the murine COL-1 antibody or the parent HuCOL-1 antibody. In yet other embodiments, a variant HuCOL-1 antibody with a CH2 domain deletion has reduced immunogenicity compared to the

murine COL-1 antibody or the parent HuCOL-1 antibody, and retains CEA binding affinity.

Effector molecules, *e.g.*, therapeutic, diagnostic, or detection moieties, can be linked to a variant HuCOL-1 antibody that specifically binds CEA, using any number of means known to those of skill in the art. Thus, a variant HuCOL-1 antibody with an amino acid substitution can have any one of a number of different types of effector molecules linked to it. In one embodiment, the variant HuCOL-1 antibody is linked to a detectable label. In some embodiments, the variant HuCOL-1 antibody is linked to a radioactive isotope, an enzyme substrate, a co-factor, a ligand, a chemiluminescent agent, a fluorescent agent, a hapten, or an enzyme. In another embodiment, the variant HuCOL-1 antibody is linked to a cytotoxin. In other embodiments, the variant HuCOL-1 antibody is linked to a chemotherapeutic drug, a radioactive isotope, a bacterially-expressed toxin, a virally-expressed toxin, or a venom protein. In yet other embodiments, the variant HuCOL-1 antibody is linked to a cytokine. Specific, non-limiting examples of cytokines are IL-2, IL-4, IL-10, TNF-alpha and IFN-gamma. In some embodiments, the variant HuCOL-1 antibody is linked to an effector molecule by a covalent or non-covalent means.

Pharmaceutical Compositions and Therapeutic Methods

Pharmaceutical compositions are disclosed herein that include a variant HuCOL-1 monoclonal antibody, such as HuCOL-1^{61}H, HuCOL-$1^{24,25,27}$L, or HuCOL-$1^{24,25,27}$L/^{61}H, and can be formulated with an appropriate solid or liquid carrier, depending upon the particular mode of administration chosen. In addition, a variant HuCOL-1 monoclonal antibody linked to an effector molecule (*i.e.*, toxin, chemotherapeutic drug, or detectable label) can be prepared in pharmaceutical compositions.

The pharmaceutically acceptable carriers and excipients useful in this disclosure are conventional. For instance, parenteral formulations usually comprise injectable fluids that are pharmaceutically and physiologically acceptable fluid vehicles such as water, physiological saline, other balanced salt solutions, aqueous dextrose, glycerol or

the like. Excipients that can be included are, for instance, other proteins, such as human serum albumin or plasma preparations. If desired, the pharmaceutical composition to be administered can also contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

The dosage form of the pharmaceutical composition will be determined by the mode of administration chosen. For instance, in addition to injectable fluids, topical, inhalation, oral and suppository formulations can be employed. Topical preparations can include eye drops, ointments, sprays and the like. Inhalation preparations can be liquid (e.g., solutions or suspensions) and include mists, sprays and the like. Oral formulations can be liquid (e.g., syrups, solutions or suspensions), or solid (e.g., powders, pills, tablets, or capsules). Suppository preparations can also be solid, gel, or in a suspension form. For solid compositions, conventional non-toxic solid carriers can include pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art.

The pharmaceutical compositions that include a variant HuCOL-1 monoclonal antibody, can be formulated in unit dosage form, suitable for individual administration of precise dosages. In addition, the pharmaceutical compositions may be administered as an immunoprophylactic in a single dose schedule or as an immunotherapy in a multiple dose schedule. A multiple dose schedule is one in which a primary course of treatment may be with more than one separate dose, for instance 1-10 doses, followed by other doses given at subsequent time intervals as needed to maintain or reinforce the action of the compositions. Treatment can involve daily or multi-daily doses of compound(s) over a period of a few days to months, or even years. Thus, the dosage regime will also, at least in part, be determined based on the particular needs of the subject to be treated and will be dependent upon the judgement of the administering practitioner. In one specific, non-limiting example, a unit dosage can be about 0.1 to about 10 mg per patient per day. Dosages from about 0.1 up to about 100 mg per patient per day may be used, particularly if the agent is administered to a secluded site

and not into the circulatory or lymph system, such as into a body cavity, into a lumen of an organ, or directly into a tumor. In one embodiment, about 10 mCi of a radiolabeled variant HuCOL-1 monoclonal antibody is administered to a subject. In other embodiments, about 15 mCi, about 20 mCi, about 50 mCi, about 75 mCi or about 100 mCi of a radiolabeled variant HuCOL-1monoclonal antibody is administered to a subject. The amount of active compound(s) administered will be dependent on the subject being treated, the severity of the affliction, and the manner of administration, and is best left to the judgment of the prescribing clinician. Within these bounds, the formulation to be administered will contain a quantity of the active component(s) in amounts effective to achieve the desired effect in the subject being treated.

The compounds of this disclosure can be administered to humans on whose tissues they are effective in various manners such as topically, orally, intravenously, intramuscularly, intraperitoneally, intranasally, intradermally, intrathecally, subcutaneously, via inhalation or via suppository. The particular mode of administration and the dosage regimen will be selected by the attending clinician, taking into account the particulars of the case (*e.g.* the subject, the disease, the disease state involved, and whether the treatment is prophylactic).

In one embodiment, a therapeutically effective amount of a variant HuCOL-1 antibody, such as HuCOL-1^{61}H, HuCOL-1^{24,25,27}L, or HuCOL-1^{24,25,27}L/^{61}H, is the amount of variant HuCOL-1 antibody necessary to inhibit further growth of a CEA-expressing tumor or suppress the growth of a CEA-expressing tumor, without eliciting a HAMA response in the patient receiving the treatment. In other embodiments, a therapeutically effective amount of variant HuCOL-1 antibody is the amount of variant HuCOL-1 antibody necessary to eliminate or reduce the size of a CEA-expressing tumor, without eliciting a HAMA response. Specific, non-limiting examples of CEA-expressing tumors are gastrointestinal, colorectal, breast, lung, and ovarian tumors. In yet another embodiment, a therapeutically effective amount of variant HuCOL-1 antibody is an amount of variant HuCOL-1 antibody that is effective at reducing a sign or a symptom of the tumor and induces a minimal immune response.

A therapeutically effective amount of a variant HuCOL-1 monoclonal antibody, such as HuCOL-1^{61}H, HuCOL-1^{24,25,27}L, or HuCOL-1^{24,25,27}L/^{61}H, can be administered in a single dose, or in several doses, for example daily, during a course of treatment. In one embodiment, treatment continues until a therapeutic result is achieved. However, the effective amount of variant HuCOL-1 antibody will be dependent on the subject being treated, the severity and type of the affliction, and the manner of administration of the therapeutic(s).

Controlled release parenteral formulations of a variant HuCOL-1 monoclonal antibody can be made as implants, oily injections, or as particulate systems. For a broad overview of protein delivery systems (see Banga, A.J., Therapeutic Peptides and Proteins: Formulation, Processing, and Delivery Systems, Technomic Publishing Company, Inc., Lancaster, PA, 1995). Particulate systems include microspheres, microparticles, microcapsules, nanocapsules, nanospheres, and nanoparticles. Microcapsules contain the therapeutic protein as a central core. In microspheres the therapeutic is dispersed throughout the particle. Particles, microspheres, and microcapsules smaller than about 1 μm are generally referred to as nanoparticles, nanospheres, and nanocapsules, respectively. Capillaries have a diameter of approximately 5 μm so that only nanoparticles are administered intravenously. Microparticles are typically around 100 μm in diameter and are administered subcutaneously or intramuscularly (see Kreuter, J., Colloidal Drug Delivery Systems, J. Kreuter, ed., Marcel Dekker, Inc., New York, NY, pp. 219-342, 1994; Tice & Tabibi, Treatise on Controlled Drug Delivery, A. Kydonieus, ed., Marcel Dekker, Inc. New York, NY, pp. 315-339, 1992).

Polymers can be used for ion-controlled release. Various degradable and nondegradable polymeric matrices for use in controlled drug delivery are known in the art (Langer, R., *Accounts Chem. Res.* 26:537, 1993). For example, the block copolymer, polaxamer 407 exists as a viscous yet mobile liquid at low temperatures but forms a semisolid gel at body temperature. It has shown to be an effective vehicle for formulation and sustained delivery of recombinant interleukin-2 and urease (Johnston *et*

al., Pharm. Res. 9:425, 1992; and Pec *et al., J. Parent. Sci. Tech.* 44:58, 1990). Alternatively, hydroxyapatite has been used as a microcarrier for controlled release of proteins (Ijntema *et al., Int. J. Pharm.* 112:215, 1994). In yet another aspect, liposomes are used for controlled release as well as drug targeting of the lipid-capsulated drug (Betageri, *et al.,* Liposome Drug Delivery Systems, Technomic Publishing Co., Inc., Lancaster, PA, 1993). Numerous additional systems for controlled delivery of therapeutic proteins are known (*e.g.,* U.S. Pat. No. 5,055,303, 5,188,837, 4,235,871, 4,501,728, 4,837,028 4,957,735 and 5,019,369, 5,055,303; 5,514,670; 5,413,797; 5,268,164; 5,004,697; 4,902,505; 5,506,206, 5,271,961; 5,254,342 and 5,534,496).

Site-specific administration of the disclosed compounds can be used, for instance by applying the variant HuCOL-1 antibody to a pre-cancerous region, a region of tissue from which a tumor has been removed, or a region suspected of being prone to tumor development. In some embodiments, sustained intra-tumoral (or near-tumoral) release of the pharmaceutical preparation that includes a therapeutically effective amount of variant HuCOL-1 antibody may be beneficial.

The present disclosure also includes therapeutic uses of variant HuCOL-1 monoclonal antibodies, such as HuCOL-1^{61}H, HuCOL-$1^{24,25,27}$L, or HuCOL-$1^{24,25,27}$L/^{61}H, that are non-covalently or covalently linked to effector molecules. In one specific embodiment, the variant HuCOL-1 monoclonal antibody is covalently linked to an effector molecule that is toxic to a tumor or cell expressing CEA. In one specific, non-limiting example, the effector molecule is a cytotoxin. In other specific, non-limiting examples the effector molecule is a radioactive isotope, a chemotherapeutic drug, a bacterially-expressed toxin, a virally-expressed toxin, a venom protein, or a cytokine. Variant HuCOL-1 monoclonal antibodies covalently linked to an effector molecule have a variety of uses. For example, a variant HuCOL-1 antibody linked to a radioactive isotope is of use in immunotherapy. A variant HuCOL-1 antibody covalently linked to a radioactive isotope is of use to localize a tumor in radioimmunoguided surgery, such that the tumor can be removed.

The present disclosure also includes combinations of a variant HuCOL-1 monoclonal antibody, such as HuCOL-1^{61}H, HuCOL-$1^{24,25,27}$L, or HuCOL-

$1^{24,25,27}L/^{61}H$, with one or more other agents useful in the treatment of tumors. For example, a therapeutically effective amount of the compounds of this disclosure can be administered in combination with effective doses of immunostimulants, anti-cancer agents, anti-inflammatory agents, anti-infectives, and/or vaccines. The term "administration in combination" or "co-administration" refers to both concurrent and sequential administration of the active agents. A subject that has a tumor, or is predisposed to the development of a tumor, will be a candidate for treatment using the therapeutic methods disclosed herein.

Diagnostic Methods and Kits

A method is provided herein for the *in vivo* or *in vitro* detection of CEA-expressing tumors or cells. An *in vivo* detection method can localize any tumor or cell that expresses CEA in a subject. In one embodiment, a variant HuCOL-1 antibody, such as HuCOL-$1^{61}H$, HuCOL-$1^{24,25,27}L$, or HuCOL-$1^{24,25,27}L/^{61}H$, is administered to the subject for a sufficient amount of time for the antibody to localize to the tumor or cell in the subject and to form an immune complex with CEA. In one embodiment, the immune complex is detected. In one specific, non-limiting example detection of an immune complex is performed by immunoscintography. Other specific, non-limiting examples of immune complex detection include radiolocalization, radioimaging, or fluorescence imaging. In another embodiment, the antibody is linked to an effector molecule. In one specific, non-limiting embodiment, the effector molecule is a detectable label. Specific, non-limiting examples of detectable labels include a radioactive isotope, an enzyme substrate, a co-factor, a ligand, a chemiluminescent agent, a fluorescent agent, a hapten, or an enzyme.

A method of detecting tumors in a subject includes the administration of a variant HuCOL-1 antibody complexed to an effector molecule, such as a radioactive isotope. In one embodiment, a variant HuCOL-1 antibody complexed to an effector molecule, such as a radioactive isotope, is administered to a subject prior to surgery or treatment. In another embodiment, a variant HuCOL-1 antibody complexed to an effector molecule, such as a radioactive isotope, is administered to a subject following

surgery or treatment. After a sufficient amount of time has elapsed to allow for the administered radiolabeled antibody to localize to the tumor, the tumor is detected. In one specific embodiment, the detection step is performed prior to surgery. In another embodiment, the detection step is performed during surgery, for example to detect the location of the tumor prior to removing it, as in radioimmunoguided surgery. In yet another embodiment, the detection step is performed after surgery to ensure the complete removal of the tumor, or to detect a recurrence of the tumor. In one specific, non-limiting example, a radiolabeled immune complex is detected using a hand-held gamma detection probe. Primary tumors, metastasized tumors, or cells expressing CEA can be detected.

In another embodiment, a variant HuCOL-1 antibody and a secondary antibody are administered to the subject for a sufficient amount of time for the variant HuCOL-1 antibody to form an immune complex with CEA on a tumor or cell, and for the secondary antibody to form an immune complex with the variant HuCOL-1 antibody. In one embodiment, the variant HuCOL-1 antibody is complexed with the secondary antibody prior to their administration to the subject. In one specific, non-limiting embodiment, the secondary antibody is linked to a detectable label. In one embodiment, the immune complex, which includes CEA, the variant HuCOL-1 antibody, and the secondary antibody linked to a detectable label, is detected as described above.

The *in vitro* detection method can screen any biological sample containing any tumor or cell that expresses CEA. Such samples include, but are not limited to, tissue from biopsies, autopsies, and pathology specimens. Biological samples also include sections of tissues, such as frozen sections taken for histological purposes. Biological samples further include body fluids, such as blood, serum, saliva, or urine. A biological sample is typically obtained from a mammal, such as a human. In one embodiment the subject has a colorectal tumor. In other embodiments, the subject has a gastrointestinal tumor, a breast tumor, a lung tumor, or an ovarian tumor. Other biological samples that can be detected by the *in vitro* detection method include samples of cultured cells that express CEA.

In one embodiment, a method is provided for detecting a CEA-expressing tumor or cell. Kits for detecting a CEA-expressing tumor or cell will typically comprise a variant HuCOL-1 antibody that specifically binds CEA. In some embodiments, an antibody fragment, such as an Fv fragment is included in the kit. In a further embodiment the antibody is an immunoconjugate. In some embodiments, the antibody is conjugated to a detectable label (for example, radioactive isotope, enzyme substrate, co-factor, ligand, fluorescent agent, hapten, enzyme, or chemiluminescent agent).

In one embodiment, a kit includes instructional materials disclosing means of use of an antibody that specifically binds CEA or fragment thereof (for example, for detection of CEA-expressing cells in a sample). The instructional materials may be written, in an electronic form (e.g. computer diskette or compact disk) or may be visual (for example, video files). The kits may also include additional components to facilitate the particular application for which the kit is designed. Thus, for example, the kit may additionally contain means of detecting a label (for example, enzyme substrates for enzymatic labels, filter sets to detect fluorescent labels, appropriate secondary labels such as a secondary antibody, or the like). In one embodiment, the kit contains a secondary antibody that is conjugated to a detectable label. The kits may additionally include buffers and other reagents, such as an antigen (for example, purified CEA) routinely used for the practice of a particular method. Such kits and appropriate contents are well known to those of skill in the art.

In one embodiment of the present invention, the diagnostic kit comprises an immunoassay. Although the details of the immunoassays may vary with the particular format employed, the method of detecting CEA or fragment thereof in a biological sample generally comprises the steps of contacting the biological sample with an antibody which specifically reacts, under immunologically reactive conditions, to CEA. The antibody is allowed to specifically bind under immunologically reactive conditions to form an immune complex, and the presence of the immune complex (bound antibody) is detected directly or indirectly.

The invention is illustrated by the following non-limiting Examples.

EXAMPLES

EXAMPLE 1

Construction of Humanized COL-1 Variants

Synthetic oligonucleotides

The long overlapping oligomers and oligonucleotide primers used for DNA amplifications were supplied by Lofstrand Labs. (Gaithersburg, MD) and Midland Certified Reagent Company (Midland, TX). The sequences of the four primers that were used to generate DNA fragments encoding the V_H and V_L domains of the murine COL-1 monoclonal antibody were as follows:

5' V_H (SEQ ID NO:1):

5'-AGT*AAGCTT*CCACCATGGAGTGGTCCTGGGTCTTCCTCTTCTT

CCTGTCCGTGACTACTGGAGTGCACTCCGAGGTTCAGCTGCAGCA-3'

3' V_H (SEQ ID NO:2):

5'-CGAT*GGGCCC*GTAGTTTTGGCAGAGGAGACGGCGACCG-3'

5' V_L (SEQ ID NO:3):

5'-TAGC*AAGCTT*CCACCATGGATAGCCAGGCCCAGGTGCTCAT

GCTCCTGCTGCTGTGGGTGAGCGGCACATGCGGCGACATTGTGCTGACACA-3'

3' V_L (SEQ ID NO:4):

5'-TGCAG*CCGCGG*CCCGTTTGATTTCCAGCTTGG-3'

Each of the above 5' primers (SEQ ID NO:1 and SEQ ID NO:3) carries a *Hind* III site followed by a sequence encoding a signal peptide. The 3' V_H primer (SEQ ID NO:2) carries an *Apa* I, while the 3' V_L primer (SEQ ID NO:4) has a *Sac* II site. The

four 119- to 133- base pair (bp)-long oligonucleotides that were used to generate each of the V_H and V_L genes of HuCOL-1 are shown by long arrows in FIG. 1. The sequence of the 20- to 21- bp-long end primers used for DNA amplification were as follows:

5' V_H (SEQ ID NO:5):

5'-CGT*AAGCTT*CCACCATGGAG-3'

3' V_H (SEQ ID NO:6):

5'-T*GGGCCC*TTGGTGGAGGCTGA-3'

5' V_L (SEQ ID NO:7):

5'-GC*AAGCTT*CCACCATGGATA-3'

3' V_L (SEQ ID NO:8):

5'-TGCAG*CCGCGGT*ACGTTTGAT-3'

The 5' primers (SEQ ID NO:5 and SEQ ID NO:7) carry a *Hind* III site. While a site for *Apa* I has been incorporated in the 3' V_H primer (SEQ ID NO:6), the sequence for the *Sac* II site has been included in the 3' V_L primer (SEQ ID NO:8). The sequences of two additional mutagenic primers (supplied by Milligen/Bioresearch, Burlington, VT) that were used for the generation and amplification of the genes encoding the V domains of HuCOL-1 variants are as follows:

3' V_H (SEQ ID NO:9):

5'-TGCCCTGGAACTTCTGGGCATATTCAGTA-3'

3' V_L (SEQ ID NO:10):

5'-GCACTGACACTTTGGCTGGACTTGCAGTTGATGGTGGCCCCTC-3'

The sequences of SEQ ID NOs:1-10 that are recognized by the restriction endonucleases are in italics, whereas the residues that introduce mutagenic changes are underlined.

DNA amplification

All PCRs were carried out in a final volume of 100 µl of PCR buffer containing 200 µM of dNTPs, 3 units of *Taq* polymerase (Boehringer Mannheim, Indianapolis, IN), 0.2 µM each of the end primers and 100 ng of DNA template. Initial denaturation at 94°C for 2 minutes was followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute. It was followed by a final primer extension step at 72°C for 10 minutes.

The V_L and V_H genes of the HuCOL-1 were synthesized by the overlap extension PCR (FIG. 1) that has previously been described (Kashmiri *et al. Hybridoma* 14:461, 1995). Primer induced mutagenesis was carried out by a dual step PCR as described by Landt et al. (Landt *et al. Gene* 96:125, 1990). The first PCR was carried out in a final volume of 100 µl containing 10 ng of template, 20 pmol of each primer, 100 µM of dNTPs and 5 units of *Taq* Polymerase (Gibco BRL, Gaithersburg, MD). Twenty-five cycles of denaturing step at 94°C for 1 minute, primer annealing step at 50°C for 2 minutes and polymerization step at 72°C for 2 minutes were followed by a final primer extension step for 15 minutes at 72°C. In the second PCR, the dNTP concentration was increased to 200 µM, and PCR consisted of 30 cycles of denaturation (94°C, 1 minute), primer annealing (45°C, 2 minutes) and extension (72°C, 2 minutes) with a final extension for 10 min at 72°C.

Isolation of COL-1 H- and L-chains

The genes encoding the L chain and the Fd region of the H chain of mAb COL-1 were generated by repertoire cloning methodology using the suggested sets of the 5' and 3' primers for the PCR amplification of the murine κ L chain and the murine H chain Fd. (Kang, *et al., Methods: A Companion to Methods in Enzymology.* 2:111, 1991). cDNA synthesized from total RNA extracted from COL-1 hybridoma (Muraro *et al., Cancer Res.* 45:5769, 1985) was used as a template. Since mAb COL-1 is a murine IgG2a, the 3' primer used to amplify the Fd region of the H chain was specific

to the 3'-end of the IgG2a C_H1 region. The cloned genes were sequenced (FIG. 2) before the phagemids were used as templates for the subsequent PCR amplification.

Comparison of genes encoding V_L and V_H of murine and COL-1and human mAbs; designing genes encoding humanized V_L and V_H of Humanized COL-1

Murine COL-1 was humanized by grafting the CDRs of the L and H chains onto the V_L and V_H frameworks of the VJI'CL and MO30 human Abs, respectively, while retaining those framework residues that were deemed essential for preserving the structural integrity of the combining site (Jones *et al., Nature* 321:522, 1986; Riechmann *et al., Nature* 332:323, 1988; Verhoeyen and Riechmann *Bioessays* 8:74, 1988). The Ig CDRs have been defined as comprising residues 31-35b, 50-65, and 95-102 in the H chain and residues 24-34, 50-56, and 89-97 in the L chain (Kabat *et al.*, *Sequence of proteins of immunological interests*, 5th Ed. U.S. Department of Health and Human Services, National Institutes of Health, Bethesda, MD (NIH Publication no. 91-3242) 1991). The framework residues that were deemed critical were identified on the basis of the atomic coordinates of the Abs of known structures available in the database (see, for example, Padlan, *Mol. Immunol.* 31:169, 1994). The human Ab sequences that are most similar to mCOL-1 are VJI'CL (Klobeck *et al., Nucleic Acids Res.* 13:6515, 1985.) (GenBank Accession Number Z00022) for V_L and MO30 (Larrick *et al., Biochem. Biophys. Res. Commun.* 160:1250, 1989) (GenBank Accession Number A32483) for V_H. The alignments of the V_L sequences of mCOL-1 and VJI'CL, and the V_H sequences of mCOL-1 and MO30 are shown in FIG. 3. Also indicated in FIG. 3 are the locations of the framework residues that are critical for Ag binding. The humanization protocols for the V_L and V_H genes, shown in FIG. 3, are based on putting the CDR sequences of mAb COL-1 together with the frameworks of the human V_L and V_H templates while replacing some of the human framework residues with those murine framework residues that may be critical for Ag binding.

A nucleotide sequence was then deduced from the amino acid sequence of each of the designed humanized V_L and V_H domains. The nucleotide sequences were refined to provide high frequency usage of codons and by eliminating, with the help of

programs FOLD and MAPSORT (Devereux *et al., Nucleic Acids Res.* 12:387, 1984), any self-annealing regions and any sites for restriction endonucleases that might complicate cloning of the designed genes in the desired vectors. Using the four overlapping oligonucleotides (shown by long arrows in FIG. 1) that encompassed, on alternating strands, the entire sequence of either the V_L or V_H region and its leader, and the respective end primers described in earlier, DNA fragments encoding the humanized V_L or V_H regions were generated and amplified by overlap extension PCR technique (Kashmiri *et al., Hybridoma* 14:461, 1995). The humanized V_L or V_H regions thus generated were extended to the *Apa* I and *Sac* II sites located 10 bp and 17 bp downstream from the 5' end of the C regions of the H and L chains, respectively. The PCR products were cloned in pBSc to generate pBScHuCOL-1V_L and pBScHuCOL-1V_H constructs. The inserts were sequenced to check the fidelity of the PCR products.

Generation of Genes Encoding Variants of Humanized COL-1 V_L and V_H Domains

Genes encoding the humanized V_L and V_H domains of the variants HuCOL-1^{24,25,27}L and HuCOL-1^{61}H were generated by primer-induced mutagenesis, using pBScHuCOL-1V_L and pBScHuCOL-1V_H constructs, respectively, as templates. Variant HuCOL-1^{61}H was generated by replacing residue 61 of mCOL-1 HCDR2 (numbering convention of Kabat *et al.* (*Sequence of proteins of immunological interests*, 5th Ed. U.S. Department of Health and Human Services, National Institutes of Health, Bethesda, MD (NIH Publication no. 91-3242) 1991)) with the corresponding residue of the human mAb MO30 HCDR2 (FIG. 3, Table 1). For the generation of the HuCOL-1^{24,25,27}L variant, residues 24, 25 and 27 of mCOL-1 LCDR1 were replaced with the corresponding residues in LCDR1 of the human mAb VJI'CL (FIG. 3, Table I). The V region sequences were synthesized by a dual step PCR procedure according to Landt *et al.* (*Gene* 96:125, 1990) as described in Example 1. For each L and H chain V region, the mutagenic primer, containing the desired nucleotide changes in the targeted CDR, was used as a 3' primer, while a 20-mer-end primer served as a 5' primer. The resulting PCR product was gel purified and used as a 5' primer for the

subsequent step of the PCR in which a 21-mer oligonucleotide was utilized as a 3' primer. The PCR products of the variants were cloned in pBSC vector and sequenced.

EXAMPLE 2

Expression of Recombinant COL-1 Antibodies in Insect Cells

Expression vectors

Two different baculovirus transfer vectors, pAcUW51 (Pharmingen, San Diego, CA) and pBAC4x-1 (Novagen, Madison, WI), have been utilized for generating the recombinant viruses and the subsequent co-expression of the Ig H and L chains. PAcUW51 vector contains the p10 and polyhedrin (*polh*) promoters placed in opposite orientation. One of the target genes can be cloned at the *BamH* I site located downstream of the *polh* promoter, while the other gene can be driven by the p10 promoter by inserting the gene either at *Bgl* II or *EcoR* I site located 3' to the promoter. The vector carries the f1 origin of replication. Co-transfection of insect cells with pAcUW51 and the baculovirus BaculoGold DNA (Pharmingen) rescues the lethal deletion in this DNA and results in the production of 99% of viable recombinant virions expressing the target gene. The pBAC4x-1 baculovirus transfer plasmid is designed for cloning and co-expression of up to four target genes. The plasmid contains two of each of the *polh* and p10 promoters, with a unique cloning site placed downstream of each promoter. The homologous promoters are placed in opposite orientation to minimize recombination. The plasmid is compatible with baculovirus DNA, BacVector2000 (Novagen).

Assembly of the V and C region genes and generation of expression constructs

To generate constructs encoding the chimeric H and L chains of mAb COL-1, the V region sequences of the H and L chain genes were PCR amplified using the phagemid constructs of the cDNAs encoding the Fd and the L chain of mCOL-1 as templates. Primers with nucleic acid sequences set forth as SEQ ID NO:3 and SEQ ID NO:4 were used as forward and reverse primers, respectively, to amplify a 420 bp

sequence encoding the V_L domain along with the signal peptide located upstream. The 3' primer was designed to extend the 3'-end of the amplified sequence to a unique *Sac* II site located 10 bp downstream from the start of the human κ C region. A DNA fragment encoding the human κ C region was excised from a pre-existing construct pLNCXHuCC49HuK (Kashmiri *et al. Hybridoma* 14:461, 1995) by *Sac* II/*Cla* I treatment. The construct carried an *EcoR* I site immediately upstream of *Cla* I site. The V and the C regions of the L chain were joined to the *Hind* III/*Cla* I linearized pBluescript II S/K$^+$ (pBSc) plasmid (Stratagene, La Jolla, CA) by three-way ligation. Taking advantage of an *EcoR* I site upstream of the *Hind* III site in pBSc, the entire L chain sequence was then released from the construct by *EcoR* I digestion and inserted into the baculovirus expression vector pAcUW51 at the *EcoR* I site located downstream from the p10 promoter (FIG. 4A).

For the assembly of the chimeric H chain, a 460 bp sequence encoding the V_H domain and its leader peptide was PCR amplified using primers, with nucleic acid sequences as set forth as SEQ ID NO:1 and SEQ ID NO:2, as the 5' and 3' primers, respectively. The design of the 3' primer facilitated amplification of the V_H sequence to extend to the *Apa* I site located 17 bp downstream from the start of the C_H1 domain. To assemble the V and C regions, an *Apa* I/*Cla* I DNA fragment carrying the human γ1 C region was excised from a pre-existing construct pLgpCXHuCC49HuG1 (Kashmiri *et al. Hybridoma* 14:461, 1995). The *Apa* I/*Cla* I fragment along with the 460 bp PCR product were inserted into the *Hind* III/*Cla* I linearized pBSc. The DNA encoding the entire H chain was released by *Hind* III/*Cla* I treatment of the pBSc construct. The termini of the target DNA were filled in using Klenow fragment of the DNA polymerase, and the DNA fragment was subcloned in the L-chain construct of pAcUW51 at the blunt-ended *BamH* I site located downstream of the *polh* promoter (FIG. 4A).

Similar DNA manipulations as those described for cCOL-1 were conducted to assemble the humanized and the variant V regions and their respective C regions into pBSc for the subsequent subcloning in baculovirus expression vector, pBAC4x-1. After

joining the V regions of the H and L chains to their respective C regions in pBSc, the assembled L chain of the HuCOL-1 or its variant was released from the pBSc construct and cloned at the *EcoR* I site, downstream from the p10 promoter (FIG. 4B). The entire H chain of the HuCOL-1 or its variant was excised from its pBSc construct by *Hind* III/*Xho* I treatment and it was cloned unidirectionally in the L-chain construct of pBAC4x-1 at the *Hind* III/*Xho* I site, downstream of *polh* promoter (FIG. 4B). Three expression constructs were generated; one containing the variant L chain and the parental humanized H chain (HuCOL-$1^{24,25,27}$L), the other containing the variant H chain and the parental humanized L chain (HuCOL-1^{61}H), while the third carried variants of both the L and H chains (HuCOL-$1^{24,25,27}$L/^{61}H) (Table I).

EXAMPLE 3

Production and Purification of Recombinant Antibodies

Development of transfectomas

Serum-free-adapted Sf9 insect cells (Gibco BRL) were cultured at 27°C in Sf900-II medium (Gibco BRL) with 50 μg/ml of gentamicin. To develop transfectomas secreting cCOL-1, insect cells were co-transfected with the pAcUW51 derived expression construct and the linearized BaculoGold Baculovirus DNA. Transfectomas producing HuCOL-1 and its variants were generated by transfecting insect cells with one of the pBAC4x-1 derived expression constructs and the linearized BacVector2000 Baculovirus DNA. A cationic liposome-mediated system DOTAP (Boehringer Mannheim) was used for all transfections. After six days, the supernatants containing the recombinant virus were harvested and screened for Ig expression and Ag binding by ELISA. Viral plaques were generated by infecting Sf9 cells (2×10^6 cells/60-mm dish) with recombinant virus followed by overlaying the infected cells with 0.5% baculovirus agarose (Invitrogen, Carlsbad, CA). Individual viral plaques were isolated and expanded for three rounds by infecting increasingly larger number of freshly seeded monolayers of Sf9 cells, using the highest producing clone as a source of inoculum each time. Supernatants harvested from each round were assayed for Ag binding by ELISA

(Bei *et al. J. Immunol. Methods* 186:245, 1995). The titer of recombinant virus was determined by plaque assay. To produce the recombinant protein, 6 x 10^8 Sf9 cells were infected with the supernatant at a multiplicity of infection of 5.

Purification of recombinant Abs

Three days after infection, the supernatants were collected and centrifuged at 2,000 × g for 10 minutes to remove cellular debris. After adding Tris-HCl pH 7.5 to a final concentration of 20 mM and incubating at 4°C for 2 hours, a second centrifugation was performed at 10,000 × g to remove any contaminating proteins. The supernatant was then loaded on a protein G agarose column (Gibco BRL). The bound protein was eluted from the column with 0.1 M glycine hydrochloride, pH 2.5 and the pH of the eluted material was immediately adjusted to 7.4 with 1.0 M Tris pH 8.0. The protein was concentrated using Centricon 30 (Amicon, Beverly, MA) and dialyzed in PBS buffer using Slide-A-Lyzer cassette (Pierce, Rockford, IL). The protein concentration was determined by the method of Lowry et al. (Lowry, *et al., J. Biol. Chem.* 193:265, 1951) and the purity of the eluted proteins was evaluated by SDS-PAGE, under reducing and non-reducing conditions, using pre-cast 4-20% Tris-glycine gel (Novex, San Diego, CA). The protein bands were visualized by Coomassie blue staining (Novex).

The apparently lower Ag-binding reactivities of the variant mAbs HuCOL-$1^{24,25,27}$L and HuCOL-$1^{24,25,27}$L/^{61}H than those of cCOL-1, HuCOL-1 and the variant HuCOL-1^{61}H could be attributed either to any possible detrimental effect of genetic manipulations of the combining site of the secreted Abs or to some structural abnormality of the expressed Ig molecules. The latter may be detected on SDS-PAGE by a change in size or mobility of the molecules. To this end, the purified Abs from the culture supernatants and the murine mAb COL-1 were analyzed by SDS-PAGE. The gel profile under non-reducing conditions (FIG. 5A) showed that the mobility of all five recombinant Abs was identical to that of mCOL-1 mAb that has an molecular weight of approximately 160 kDa. Under reducing conditions, all the recombinant COL-1 Abs,

like that of mCOL-1, yielded two protein bands of approximately 25-28 kDa and 50-55 kDa (FIG. 5B). These molecular masses are in conformity with those of the Ig L and H chains. The results of the SDS-PAGE analysis, together with the ELISA assay for CEA reactivity of the serially diluted antibodies, suggest that the reduced Ag reactivity of the variants HuCOL-$1^{24,25,27}$L and HuCOL-$1^{24,25,27}$L/^{61}H may be due to some detrimental effect of the amino acid substitutions in the combining site of HuCOL-1.

ELISA

ELISA assays were carried out by coating 96-well polyvinyl microtiter plates with CEA (100 ng/well) (Research Diagnostic Inc., Flanders, NJ) or with Fcγ-fragment-specific goat anti-human IgG (100 ng/well) (Jackson Immunoresearch Lab, West Grove, PA). Anti-human IgG or the CEA-coated plates were used to test for the production of Ig by the insect cells or to assess its Ag reactivity, respectively. The plates were blocked with 5% BSA in PBS for 1 hour at 37°C and then washed with 1% BSA in PBS. Fifty microliters of culture supernatants were loaded in duplicate wells. After 1hour incubation at 37°C and washing with 1% BSA in PBS, 100 µl of peroxidase-conjugated anti-human IgG (Fcγ-fragment specific) diluted 1:3000 in 1% BSA in PBS was added per well. The plates were incubated for another hour at 37°C. They were washed prior to the addition of 100 µl of freshly prepared substrate (H_2O_2) mixed with o-phenylene diamine hydrochloride as a chromogen (Sigma, St. Louis, MO). The colorimetric reaction was allowed to proceed for 10 minutes at room temperature in the dark, before it was terminated by the addition of 50 µl of 4N H_2SO_4 per well. The absorbance was read at 490 nm.

All the transfectants and the viral plaques, generated by infecting Sf9 cells with the infectious supernatants, were found to be positive for Ig production as assayed by ELISA. Results of an ELISA assay for Ag binding also showed that all culture supernatants and the viral plaques were reactive with CEA, albeit with varying degrees. To examine whether the different constructs were expressing comparable levels of Ig molecules, viral plaques were expanded and a large batch of Sf9 cells were freshly

infected, at a multiplicity of infection of 5, with infectious supernatant derived from the highest producing clone of each construct, and the infected cells were cultured under identical conditions. The secreted Abs were purified from equal volumes of the culture supernatants. The concentration of the secreted Abs was comparable (2 to 3 μg/ml) in culture supernatants of all five transfectants. ELISA assays were carried out using serial dilutions of the purified recombinant Abs. Results of the assays suggested that mAbs HuCOL-$1^{24,25,27}$L and HuCOL-$1^{24,25,27}$L/^{61}H had lower CEA-binding reactivity than those of cCOL-1, HuCOL-1 and the variant HuCOL-1^{61}H mAbs.

EXAMPLE 4

Competition Radioimmunoassay

The relative Ag binding of the mCOL-1 and the recombinant antibodies derived from it were determined using competition radioimmunoassay (RIA). Twenty-five μl of serial dilutions of the Abs to be tested as well as the mCOL-1, prepared in 1% BSA in PBS, were added to microtiter plates containing 200 ng of CEA saturated with 5% BSA in PBS. ^{125}I-labeled mCOL-1 or ^{125}I-labeled HuCOL-1 (100,000 cpm in 25 μl) was then added to each well. After an overnight incubation at 4°C, the plates were washed and counted in a γ-scintillation counter. The relative affinity constants were calculated by a modification of the Scatchard method (Frankel and Gerhard, *Mol. Immunol.* 16:101, 1979).

When serial dilutions of unlabeled Abs (murine, chimeric COL-1 (cCOL-1), humanized COL-1 and its variants) were used to compete with the binding of ^{125}I-mCOL-1 (FIG. 6A) or ^{125}I-HuCOL-1 (FIG. 6B) to CEA, all the COL-1 derived recombinant Abs, like the mCOL-1, were able to completely inhibit the binding of ^{125}I-mCOL-1 and ^{125}I-HuCOL-1 to CEA. The competition profiles of cCOL-1, HuCOL-1 and the variant HuCOL-1^{61}H were comparable to that of the mCOL-1. In contrast, the competition profiles of mAbs HuCOL-$1^{24,25,27}$L and HuCOL-$1^{24,25,27}$L/^{61}H, although of similar slopes as that of the mCOL-1 mAb, were shifted to the right. The values of the

relative affinity constants (K_a) of cCOL-1, HuCOL-1 and HuCOL-1^{61}H mAbs, calculated from the linear parts of the competition curves in FIG. 6B, were 3.45 x 10^8 M^{-1}, 2.82 x 10^8 M^{-1} and 2.64 x 10^8 M^{-1}, respectively. These relative affinities are approximately 1.5-2.0-fold less than that of mCOL-1 (5.17 x 10^8 M^{-1};Table I). The relative K_a values of HuCOL-$1^{24,25,27}$L and HuCOL-$1^{24,25,27}$L/^{61}H were 1.2 x 10^8 M^{-1} and 1.03 x 10^8 M^{-1}, respectively, approximately 4.3-5.0-fold lower than that of mCOL-1 (Table I).

EXAMPLE 5

Flow cytometric analysis

A previously described method (Guadagni *et al., Cancer Res.* 50:6248, 1990) has been used for FACS analysis. To evaluate the ability of HuCOL-1 and its variants to bind to cell-surface CEA, 1x10^6 retrovirally transduced MC38 cells expressing CEA (Robbins *et al., Cancer Res.* 51:3657, 1991) were resuspended in cold Ca^{++} and Mg^{++} free Dulbecco's PBS and incubated with the mCOL-1 derived Abs for 30 minutes on ice. A human IgG was used as an isotype control. After one washing cycle, the cell suspension was stained with FITC-conjugated mouse anti-human Ab (Pharmingen) for 30 minutes on ice. A second washing cycle was performed and then the samples were analyzed with a FACScan (Becton Dickinson, Mountain View, CA) using CellQuest for Macintosh. Data from analysis of 10,000 cells were obtained.

No significant differences were found in the mean fluorescence intensity, or in the percentage of cells that were reactive with HuCOL-1 and its variants (FIG. 7). The percentages of gated cells, calculated after exclusion of irrelevant binding, were indeed between 54 and 56, while the mean fluorescence intensities were between 15 and 16 when 1 μg of each Ab was used (FIG. 7A). Similar FACS profiles were observed when 0.5 μg of each antibody was used (FIG. 7B).

EXAMPLE 6

Reactivity of HuCOL-1 and HuCOL-1 Variants to Patients' Sera

Stored patients' sera, from a phase I clinical trial (Yu *et al., J. Clin. Oncol.* 14:1798, 1996), which involved the administration of ^{131}I-mCOL-1 to gastrointestinal carcinomas patients, were used to assess sera reactivity of the mCOL-1 derived Abs. Several sera were tested for the presence of anti-V region Abs to mAb COL-1. The sera, however, contain circulating Ag and anti-murine Fc Abs, which could interfere with the binding of mAb COL-1 and its derivative Abs to the anti-V region Abs. To circumvent this problem, the circulating CEA and anti-murine Fc Abs were removed by sequential pre-adsorption of the sera with a purified mCOL-4 mAb that reacts with epitopes of CEA different from the one recognized by mCOL-1. Murine COL-6 has the same isotype as that of mCOL-1. For pre-adsorption, serum samples were added to mCOL-6 coupled to Reacti-gel according to the method of Hearn et al. (Hearn *et al., J. Chromatogr.* 185:463, 1979). The mixtures were incubated overnight at 4°C with end-to-end rotation and centrifuged at 1000 x *g* for 5 min. Pre-adsorption was repeated until the supernatants displayed no detectable anti-murine Fc activity. To detect anti-V region Abs by Surface Plasmon Resonance (SPR), the pre-adsorbed serum was used as a mobile reactant. Proteins were immobilized on carboxymethylated dextran CM5 chips (Biacore, Piscataway, NJ) by amine coupling using standard procedure (Johnsson *et al., Anal. Biochem.* 198:268, 1991; Schuck et al., Measuring protein interactions by optical biosensors. In *Current Protocols in Protein Science*, Vol. 2. J. E. Coligan, B. M. Dunn, H. L. Ploegh, D. W. Speicher, and P. T. Wingfield, eds. John Wiley & Sons, New York, NY, p. 20.2.1, 1999). HuCOL-1 was immobilized on the surface of flow cell 1, while the surface of flow cell 2 was coated with an unrelated protein, rabbit gamma globulin (Biorad, Hercules, CA).

The reactivity of COL-1 variants to anti-V region antibodies was determined using a recently developed SPR-based competition assay (De Pascalis *et al., J. Immunol.* In Press). Competition experiments were performed at 25 °C using a CM5 sensor chip containing either mCOL-1 or HuCOL-1 in flow cell 1 and rabbit gamma globulin (Biorad), as a reference, in flow cell 2. Typically, mCOL-1, HuCOL-1 or its

variants, were used at different concentrations, to compete with the Ab immobilized on the sensor chip for binding to serum anti-V region Abs. Patient's serum with or without the competitor (mCOL-1, HuCOL-1 or its variants) was applied across the sensor surface using a recently developed sample application technique (Abrantes *et al., Anal. Chem.* 73:2828, 2001) at the unidirectional flow of 1 µl/min. After the binding was measured for 1000 sec, the samples were washed from the surfaces with running buffer using a flow rate of 100 µl/min, and the surfaces were regenerated with 10 mM glycine (pH 2.0) for the HuCOL-1 sensor chip or HCl (pH 2.3) for the muCOL-1 sensor chip. The percent binding at each Ab concentration was calculated as follows: % binding = [slope of the signal obtained with competitor (serum + mCOL-1, HuCOL-1 or HuCOL-1 variants) / slope of the signal obtained without competitor (serum only)] x 100. IC_{50} for each antibody, the concentration required for 50% inhibition of the binding of the serum to either mCOL-1 or HuCOL-1, was calculated.

To assess the potential immunogenicity of the mCOL-1, HuCOL-1 and its variants in patients, the Abs were characterized for their reactivity to sera from gastrointestinal carcinoma patients who were administered ^{131}I-mCOL-1 in a phase I clinical trial (Yu *et al., J. Clin. Oncol.* 14:1798, 1996). As described above, any circulating CEA and anti-murine Fc Abs were removed from the sera by immunoadsorption with mCOL-4, a murine anti-CEA Ab of IgG_{2a} isotype that reacts with a CEA epitope different from the one recognized by mAb COL-1. Pre-adsorbed sera were tested for the presence of anti-V region Abs to mAb COL-1. Specific binding profiles of HuCOL-1 to the sera from patients EM, JS and MB, presented in FIG. 8, show that all three sera have antibodies against the variable regions of mCOL-1. Sera reactivity of HuCOL-1 and HuCOL-1 variants was determined by their ability to compete with mCOL-1 or HuCOL-1 immobilized on a sensor chip for binding to the anti-variable region antibodies to mCOL-1 present in the serum. IC_{50}, the concentration of the competitor Ab required for 50% inhibition of the binding of mCOL-1 or HuCOL-1 to the patient's serum, was calculated by plotting the percent inhibition as a function of competitor concentration. A higher IC_{50} value indicates a decreased reactivity to the serum suggesting potentially reduced immunogenicity of the

Abs in patients. FIG. 9 shows the competition profiles generated by HuCOL-1 and its variants when they were used to compete with the HuCOL-1 immobilized on the sensor chip for binding to the anti-V region Abs to COL-1 present in the sera of patients EM (FIG. 9A), JS (FIG. 9B), and MB (FIG. 9C). The competition profiles were used to calculate IC_{50} values that are presented in Table 2.

Table 2 Concentrations of competitor antibody required for half-maximal inhibition (IC_{50}) of binding of patients' sera to HuCOL-1

Competitor Antibody	Patient EM (nM)	Patient JS (nM)	Patient MB (nM)
HuCOL-1	6.6	2.0	5.5
^{24,25,27}L	10.7	2.5	15.2
^{61}H	4.9	2.8	10.5
^{24,25,27}L/^{61}H	9.1	4.1	14.4

For serum MB, the IC_{50} values of all three variants are 2- to 3-fold higher than that of HuCOL-1. Studies with the serum from patient EM show that the variants HuCOl-1^{24,25,27}L and HuCOL-1^{24,25,27}L/^{61}H have 50% higher IC_{50} values, while the variant HuCOL-1^{61}H has significantly lower IC_{50} value, than that of HuCOL-1. For the serum from patient JS, the IC_{50} of the variant HuCOL-1^{24,25,27}L/^{61}H is twice as much as that of HuCOL-1, while the IC_{50} values of the variant HuCOL-1^{61}H and HuCOL-1^{24,25,27}L are comparable to that of parental HuCOL-1.

When mCOL-1 was immobilized on the sensor chip and mCOL-1, HuCOL-1 and the variant HuCOL-1^{24,25,27}L/^{61}H were used to compete with it for binding to the anti-V region Abs in the serum of patient MB, the competition profiles shown in FIG. 10 were generated. The data show that the concentration of HuCOL-1 required for 50% inhibition of the binding of the patient's serum to mCOL-1 is approximately 3-fold

higher than that of mCOL-1, whereas the concentration of the variant HuCOL-$1^{24,25,27}L/^{61}H$ required to attain 50% inhibition of the binding of mCOL-1 to the patient's serum is approximately 5.5- and 17-fold higher than that of HuCOL-1 and mCOL-1, respectively. Moreover, it should be pointed out that the slope of the competition profile of the variant HuCOL-$1^{24,25,27}L/^{61}H$ is quite different from that of mCOL-1. Indeed, there was more than 2 log differential in the concentrations of the HuCOL-$1^{24,25,27}L/^{61}H$ and mCOL-1 mAbs required for the 60% inhibition of the binding of the sera anti-V region Abs to mCOL-1 immobilized on the sensor chip (FIG. 10). Sera from two other patients, JS and EM, were used to compare sera reactivity of mCOL-1 and HuCOL-1. While for serum JS the IC_{50} value of HuCOL-1 was approximately 6-fold higher than that of mCOL-1, it was not possible to evaluate the difference in the reactivity of the two Abs to EM serum; even 1000 nM of HuCOL-1 was unable to attain 50% inhibition of the binding of the serum to mCOL-1.

EXAMPLE 7

HuCOL-1 Variant Testing in Patients

Patients and Sample Collection

Patients with recurrent colorectal cancer are assessed to determine the maximum tolerated dose of intravenously administered ^{177}Lutetium radiolabeled HuCOL-$1^{61}H$, ^{177}Lutetium radiolabeled HuCOL-$1^{24,25,27}L$, and ^{177}Lutetium radiolabeled HuCOL-$1^{24,25,27}L/^{61}H$ (Mulligan, (1995) *Clin. Cancer Res.* 1:1447-1454). Colorectal cancer patients are given a test dose of 0.1 mg (intravenous bolus) of HuCOL-$1^{61}H$, HuCOL-$1^{24,25,27}L$, or HuCOL-$1^{24,25,27}L/^{61}H$ and are observed for 30 minutes prior to administration of the ^{177}Lu-labeled HuCOL-$1^{61}H$, ^{177}Lu-labeled HuCOL-$1^{24,25,27}L$, or ^{177}Lu-labeled HuCOL-$1^{24,25,27}L/^{61}H$. The radiolabeled antibodies are given as an intravenous infusion over the course of a one hour time interval. Blood samples are collected prior to and at the end of the infusion, as well as 0.5, 1 and 2 hours following the completion of the infusion. In addition, blood samples are collected daily over the subsequent 7 days. Patients return for a follow-up examination at 3, 6 or 8 weeks.

Blood samples are again collected during these visits. Sera are separated and stored at -20°C.

Determination of Patient Humoral Response

The sera from the patients are evaluated for the presence of human anti-murine antibodies (HAMA) in response to radiolabeled HuCOL-1^{61}H, HuCOL-$1^{24,25,27}$L, or HuCOL-$1^{24,25,27}$L/^{61}H using the SPR-based assay described in Example 8, above. The sera are pre-absorbed with an mCOL-4 monoclonal antibody that recognizes an epitope of CEA which is different from the epitope recognized by the humanized COL-1 monoclonal antibody. Pre-absorption using the COL-4 antibody removes circulating CEA and anti-murine Fc antibodies from the sera. To monitor the sera-reactivity of the anti-variable antibodies in the pre-absorbed sera, HuCOL-1^{61}H, HuCOL-$1^{24,25,27}$L, or HuCOL-$1^{24,25,27}$L/^{61}H is coated on the surface of flow cell 1 and a reference protein (HuIgG2a, bovine serum albumin, or rabbit gamma globulin) is immobilized on the surface of flow cell 2. A small, known volume of a patient serum sample us applied to each flow cell using the recently developed sample application technique described in Example 8 (Abrantes *et al.*, *Anal. Chem.* 73:2828, 2001). Sensograms to flow cell 1 and flow cell 2 are generated and the response difference between the two cells is plotted for each serum sample, thus providing a measure of the anti-variable region response against HuCOL-1^{61}H, HuCOL-$1^{24,25,27}$L, or HuCOL-$1^{24,25,27}$L/^{61}H in each particular serum sample. Results indicate that the patients' sera have a minimal anti-variable region response against the HuCOL-1^{61}H, HuCOL-$1^{24,25,27}$L, or HuCOL-$1^{24,25,27}$L/^{61}H antibodies.

This disclosure provides humanized COL-1 monoclonal antibodies. The disclosure further provides methods of diagnosing and treating tumors using these humanized COL-1 antibodies. It will be apparent that the precise details of the methods described may be varied or modified without departing from the spirit of the described disclosure. We claim all such modifications and variations that fall within the scope and spirit of the claims below.

CLAIMS

We claim:

1. A humanized monoclonal antibody comprising:

a light chain Complementarity Determining Region (LCDR)1, a LCDR2, and a LCDR3; and a heavy chain Complementarity Determining Region (HCDR)1, a HCDR2, and a HCDR3 from monoclonal antibody COL-1, wherein the LCDR1, LCDR2, LCDR3 are in a human light chain framework and wherein the LCDR1, LCDR2 and LCDR3 are in a human heavy chain framework; wherein the HCDR2 comprises an amino acid substitution of a non-ligand contact residue;

wherein the humanized COL-1 antibody retains or has increased binding affinity for carcinoembryoinc antigen (CEA) and has reduced immunogenicity, as compared to a parental antibody.

2. The humainzed antibody of claim 1, wherein the parental antibody is murine monoclonal COL-1 or HuCOL-1.

3. The antibody of claim 1, wherein the non-ligand contact residue is at position 61.

4. The monoclonal antibody of claim 3, comprising a glutamine at position 61.

5. The humanized antibody of claim 1, wherein the antibody has a relative antigen binding affinity constant for CEA of at least about 2.5×10^{-8} molar.

6. The humanized antibody of claim 1, further comprising at least one additional amino acid substitution in LCDR1, LCDR2 or LCDR3.

7. The humanized antibody of claim 6, comprising an amino acid substitution in LCDR1 at position 24, 25, 27, or a combination thereof.

8. The humanized antibody of claim 7, comprising a lysine at position 24.

9. The humanized antibody of claim 7, comprising a serine at position 25.

10. The humanized antibody of claim 7, comprising a glutamine at position 27.

11. The humanized antibody of claim 1, further comprising an effector molecule.

12. The humanized antibody of claim 11, wherein the effector molecule is a detectable label.

13. The humanized monoclonal antibody of claim 12, wherein the detectable label comprises a radioactive isotope, an enzyme substrate, a co-factor, a ligand, a chemiluminescent agent, a fluorescent agent, a hapten, or an enzyme.

14. The humanized monoclonal antibody of claim 11, wherein the effector molecule is a toxin.

15. The humanized monoclonal antibody of claim 14, wherein the toxin is a chemotherapeutic drug, a radioactive isotope, a bacterial toxin, a viral toxin, a cytokine or a venom protein.

16. An isolated nucleic acid molecule encoding the humanized monoclonal antibody of claim 1.

17. The nucleic acid of claim 16, operably linked to a promoter.

18. A vector comprising the nucleic acid of claim 17.

19. A humanized monoclonal antibody comprising:

a light chain Complementarity Determining Region (LCDR)1, a LCDR2, and a LCDR3; and a heavy chain Complementarity Determining Region (HCDR)1, a HCDR2, and a HCDR3 from monoclonal antibody COL-1, wherein the LCDR1, LCDR2, LCDR3 are in a human light chain framework and wherein the LCDR1, LCDR-2 and LCDR3 are in a human heavy chain framework; wherein the LCDR1 comprises amino acid substitutions of a non-ligand contact residue at positions 24, 25 and 27;

wherein the humanized COL-1 antibody specifically binds carcinoembryonic antigen (CEA) and has reduced immunogenicity, as compared to a parental antibody.

20. The humanized antibody of claim 19, comprising a lysine at position 24.

21. The humanized antibody of claim 19, comprising a serine at position 25.

22. The humanized antibody of claim 19, comprising a glutamine at position 27.

23. The humanized antibody of claim 19, wherein the parental antibody is murine monoclonal COL-1 or HuCOL-1.

24. The humanized antibody of claim 19, wherein the antibody has a relative antigen binding affinity constant for CEA of at least about 1.0×10^{-8} molar.

25. The humanized antibody of claim 19, further comprising at least one additional amino acid substitution in HCDR1, HCDR2 or HCDR3.

26. The humanized antibody of claim 25, comprising an amino acid substitution in HCDR2 at position 61.

27. The humanized antibody of claim 26, comprising a glutamine at position 61.

28. The humanized antibody of claim 19, further comprising an effector molecule.

29. The humanized antibody of claim 28, wherein the effector molecule is a detectable label.

30. The humanized monoclonal antibody of claim 29, wherein the detectable label comprises a radioactive isotope, an enzyme substrate, a co-factor, a ligand, a chemiluminescent agent, a fluorescent agent, a hapten, or an enzyme.

31. The humanized monoclonal antibody of claim 28, wherein the effector molecule is a toxin.

32. The humanized monoclonal antibody of claim 31, wherein the toxin is a chemotherapeutic drug, a radioactive isotope, a bacterial toxin, a viral toxin, a cytokine or a venom protein.

33. An isolated nucleic acid molecule encoding the humanized monoclonal antibody of claim 19.

34. The nucleic acid of claim 33, operably linked to a promoter.

35. A vector comprising the nucleic acid of claim 34.

36. A humanized COL-1 antibody, wherein the antibody is encoded by a nucleic acid sequence deposited as ATCC Accession Number PTA-4642, or ATCC Accession Number PTA-4643, or ATCC Accession Number PTA-4644.

37. A method of detecting a carcinoembryonic antigen (CEA)-expressing cell in a sample, comprising:

contacting the sample with the antibody of claims 1 or 19 for a sufficient amount of time to form an immune complex;

detecting the presence of the immune complex, wherein the presence of the immune complex demonstrates the presence of the CEA-expressing cell in the sample.

38. The method of claim 37, wherein the sample is a colorectal carcinoma, a gastrointestinal carcinoma, a breast carcinoma, a lung carcinoma, or an ovarian carcinoma.

39. The method of claim 37, wherein the antibody comprises a detectable label.

40. The method of claim 39, wherein the detectable label comprises a radioactive isotope, an enzyme substrate, a co-factor, a ligand, a chemiluminescent agent, a fluorescent agent, a hapten, or an enzyme.

41. A method of detecting a carcinoembryonic antigen (CEA)-expressing tumor in a subject, comprising:

administering a therapeutically effective amount of the antibody of claims 1 or 19 to the subject for a sufficient amount of time to form an immune complex;

detecting the presence of the immune complex, wherein the presence of the immune complex demonstrates the presence of the CEA-expressing tumor in the subject.

42. The method of claim 41, wherein the antibody comprises a detectable label.

43. The method of claim 42, wherein the detectable label comprises a radioactive isotope, an enzyme substrate, a co-factor, a ligand, a chemiluminescent agent, a fluorescent agent, a hapten, or an enzyme.

44. The method of claim 41, wherein the tumor is a colorectal carcinoma, a gastrointestinal carcinoma, a breast carcinoma, a lung carcinoma, or an ovarian carcinoma.

45. A method of treating a subject having a tumor that expresses carcinoembryonic antigen, comprising

administering to the subject a therapeutically effective amount of the antibody of claims 1 or 19,

thereby treating the subject.

46. The method of claim 45, wherein the administration of a therapeutically effective amount of the antibody of claims 1 or 19 does not elicit a human anti-murine antibody response in a subject.

47. The method of claim 45, wherein the tumor is a carcinoma.

48. The method of claim 45, wherein the antibody further comprises an effector molecule.

49. The method of claim 48, wherein the effector molecule is a chemotherapeutic drug, a radioactive isotope, a bacterial toxin, a viral toxin, a cytokine, or a venom protein.

50. A method of treating a subject having a carcinoembryonic antigen (CEA)-expressing tumor, comprising:

administering the antibody of claims 1 or 19 to the subject for a sufficient amount of time to form an immune complex, wherein the antibody comprises a radioactive isotope;

detecting the presence of the immune complex with a hand-held gamma counter, wherein the presence of the immune complex demonstrates the presence of the CEA-expressing tumor; and

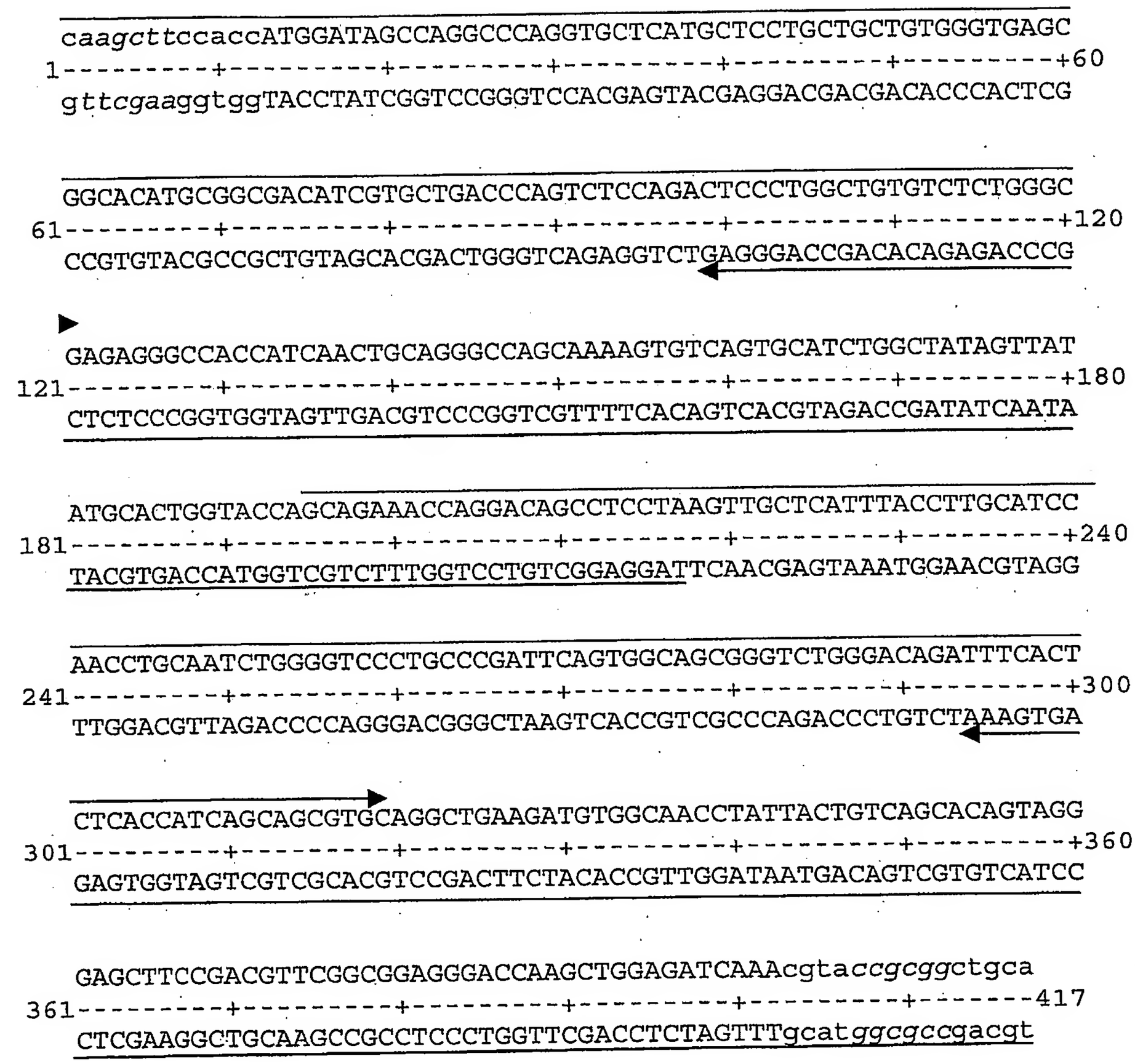
removing the tumor surgically, thereby treating the subject.

51. A pharmaceutical composition comprising a therapeutically effective amount of the antibody of claims 1 or 19 in a pharmaceutically acceptable carrier.

52. A kit, comprising a container comprising the antibody of claims 1 or 19.

53. The kit of claim 52, further comprising a container containing an antigen, a container containing a secondary antibody conjugated to a chemical compound, instructions for using the kit, or any combination thereof.

A.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gttcgaaggtggTACCTATCGGTCCGGGTCCACGAGTACGAGGACGACGACACCCACTCG
GGCACATGCGGCGACATCGTGCTGACCCAGTCTCCAGACTCCCTGGCTGTGTCTCTGGGC
61---------+---------+---------+---------+---------+---------+120
CCGTGTACGCCGCTGTAGCACGACTGGGTCAGAGGTCTGAGGGACCGACACAGAGACCCG
GAGAGGGCCACCATCAACTGCAGGGCCAGCAAAAGTGTCAGTGCATCTGGCTATAGTTAT
121---------+---------+---------+---------+---------+---------+180
CTCTCCCGGTGGTAGTTGACGTCCCGGTCGTTTTCACAGTCACGTAGACCGATATCAATA
ATGCACTGGTACCAGCAGAAACCAGGACAGCCTCCTAAGTTGCTCATTTACCTTGCATCC
181---------+---------+---------+---------+---------+---------+240
TACGTGACCATGGTCGTCTTTGGTCCTGTCGGAGGATTCAACGAGTAAATGGAACGTAGG
AACCTGCAATCTGGGGTCCCTGCCCGATTCAGTGGCAGCGGGTCTGGGACAGATTTCACT
241---------+---------+---------+---------+---------+---------+300
TTGGACGTTAGACCCCAGGGACGGGCTAAGTCACCGTCGCCCAGACCCTGTCTAAAGTGA
CTCACCATCAGCAGCGTGCAGGCTGAAGATGTGGCAACCTATTACTGTCAGCACAGTAGG
301---------+---------+---------+---------+---------+---------+360
GAGTGGTAGTCGTCGCACGTCCGACTTCTACACCGTTGGATAATGACAGTCGTGTCATCC
GAGCTTCCGACGTTCGGCGGAGGGACCAAGCTGGAGATCAAAcgtaccgcggctgca
361---------+---------+---------+---------+---------+-------417
CTCGAAGGCTGCAAGCCGCCTCCCTGGTTCGACCTCTAGTTTgcatggcgccgacgt

FIGURE 1A

B.

```
   taagcttccaccATGGAGTGGTCCTGGGTCTTCCTCTTCTTCCTGTCCGTGACTACTGGA
 1---------+---------+---------+---------+---------+---------+---------+60
   attcgaaggtggTACCTCACCAGGACCCAGAAGGAGAAGAAGGACAGGCACTGATGACCT

   GTGCACTCCGAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGGTGAAGCCTGGGGCCTCA
61---------+---------+---------+---------+---------+---------+---------+120
   CACGTGAGGCTCCACGTCGACCACGTCAGACCCCGACTCCACCACTTCGGACCCCGGAGT

   GTGAAGATGTCCTGCAAGGCATCTGGATTCAACATTAAAGACTACTATATGCACTGGGTG
121---------+---------+---------+---------+---------+---------+---------+180
   CACTTCTACAGGACGTTCCGTAGACCTAAGTTGTAATTTCTGATGATATACGTGACCCAC

   AAGCAGGCCCCTGGACAAGGGCTTGAGTGGATTGGATGGATTGATCCTGAGAATGGTGAT
181---------+---------+---------+---------+---------+---------+---------+240
   TTCGTCCGGGGACCTGTTCCCGAACTCACCTAACCTACCTAACTAGGACTCTTACCACTA

   ACTGAATATGCCCCGAAGTTCCAGGGCAAGGCCACCATGACCACGGACACGTCCACGAGC
241---------+---------+---------+---------+---------+---------+---------+300
   TGACTTATACGGGGCTTCAAGGTCCCGTTCCGGTGGTACTGGTGCCTGTGCAGGTGCTCG

   ACGGCCTACCTGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTATTACTGTAAT
301---------+---------+---------+---------+---------+---------+---------+360
   TGCCGGATGGACCTCGACTCGTCGGACTCTAGACTCCTGTGCCGGCACATAATGACATTA

   ACACGGGGTCTATCTACTATGATTACGACGCGCTGGTTCTTCGATGTCTGGGGCGCAGGA
361---------+---------+---------+---------+---------+---------+---------+420
   TGTGCCCCAGATAGATGATACTAATGCTGCGCGACCAAGAAGCTACAGACCCCGCGTCCT

   ACCCTGGTCACCGTCTCCTCAgcctccaccaagggccca
421---------+---------+---------+---------+---------459
   TGGGACCAGTGGCAGAGGAGTcggaggtggttcccgggt
```

FIGURE 1B

A.

FIGURE 2A and 2B

```
GAC ATT GTG CTG ACA CAG TCT CCT GCT TCC TTA ACT GTA TCT CTG GGG
Asp Ile Val Leu Thr Gln Ser Pro Ala Ser Leu Thr Val Ser Leu Gly

CTG AGG GCC ACC ATC TCA TGC AGG GCC AGC AAA AGT GTC AGT GCA TCT
Leu Arg Ala Thr Ile Ser Cys Arg Ala Ser Lys Ser Val Ser Ala Ser

GGC TAT AGT TAT ATG CAC TGG TAC CAA CAG AGA CCA GGA CAG CCA CCC
Gly Tyr Ser Tyr Met His Trp Tyr Gln Gln Arg Pro Gly Gln Pro Pro

AAA CTC CTC ATC TAT CTT GCA TCC AAC CTA CAA TCT GGG GTC CCT GCC
Lys Leu Leu Ile Tyr Leu Ala Ser Asn Leu Gln Ser Gly Val Pro Ala

AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACC CTC AAC ATC CAT
Arg Phe Ser Gly Ser gly Ser Gly Thr Asp Phe Thr Leu Asn Ile His

CCT GTG GAG GAG GAG GAT GCT GCA ACC TAT TAC TGT CAG CAC AGT AGG
Pro Val Glu Glu Glu Asp Ala Ala Thr Tyr Tyr Cys Gln His Ser Arg

GAG CTT CCG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA
Glu Leu Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
```

B.

```
GAG GTT CAG CTG CAG CAG TCT GGG GCA GAG CTT GTG AGG TCA GGG GCC
Glu Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Arg Ser Gly Ala

TCA GTC AAG ATG TCC TGC ACA GCT TCT GGC TTC AAC ATT AAA GAC TAC
Ser Val Lys Met Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys Asp Tyr

TAT ATG CAC TGG GTG AAG CAG AGG CCT GAA CAG GGC CTG GAG TGG ATT
Tyr Met His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu Glu Trp Ile

GGA TGG ATT GAT CCT GAG AAT GGT GAT ACT GAA TAT GCC CCG AAG TTC
Gly Trp Ile Asp Pro Glu Asn Gly Asp Thr Glu Tyr Ala Pro Lys Phe

CAG GGC AAG GCC ACT ATG ACT ACA GAC ACA TCC TCC AAC ACA GCC TAC
Gln Gly Lys Ala Thr Met Thr Thr Asp Thr Ser Ser Asn Thr Ala Tyr

CTG CAG CTC AGC AGC CTG ACA TCT GAG GAC ACT GCC GTC TAT TAC TGT
Leu Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val Tyr Tyr Cys

AAT ACA CGG GGT CTA TCT ACT ATG ATT ACG ACG CGT TGG TTC TTC GAT
Asn Thr Arg Gly Leu Ser Thr Met Ile Thr Thr Arg Trp Phe Phe Asp

GTC TGG GGC GCA GGG ACC ACG GTC GCC GTC TCC TCT
Val Trp Gly Ala Gly Thr Thr Val Ala Val Ser Ser
```

A.

```
                                        CDR1                                CDR2
COL-1 DIVLTQSPASLTVSLGLRATISC RASKSVSASGYSYMH WYQQRPGQPPKLLIY LASNLQS
VJI'CL ---M-------A----E----N- KS-Q--LYNNKN-LA ----K---------- W--TRE-
HuCOL---L-------A----E----N- RA-K--SASGYS-MH ----K---------- L--NLQ-
Variant ---L-------A----E----N- KS-Q--SASGYS-MH ----K---------- L--NLQ-

                                    CDR3
COL-1 GVPARFSGSGSGTDFTLNIHPVEEEDAATYYC QHSRELPT FGGGTKLEIK
VJI'CL ---D------------T-SSLQA--V-V--- -QYDTI-- ------V---
HuCOL---A------------T-SSVQA--V-T--- -HSREL-- ------L---
Variant ---A------------T-SSVQA--V-T--- -HSREL-- ------L---
```

B.

```
                                CDR1                CDR2
COL-1 EVQLQQSGAELVRSGASVK SCTASGFNIK DYYMH WVKQRPEQGLE IG WIDPENGDTEYAP
MO30 Q---V-----VKKP-----V--K---YTFT N---- --R-A-G-----M- I-N-SGNS-N--Q
HuCOL-E---V-----VVKP----- --K---FNIK D---- --K-A-G-----I- W-D-ENGD-E--P
Variant E---V-----VVKP----- --K---FNIK D---- -- - - ----- - W-D-ENGD-E--Q

                                    CDR3                ...
COL-1 KFQG KATMTTDTSSNTAYLQLSSLTSEDTAVYYCNT RGLSTMITTR FFDV WGAGTTVOVSS
MO30 ---- RV---R---TS-V-ME----R---------AR EK-A-T-FIITGM-Y --Q--L-T---
HuCOL---- KA---T---TS-A-LE----R---------NT RG-S-M-TTR FF-V --A--L-T---
Variant ---- KA---T---TS-A-LE----R---------NT RG-S-M-TTR FF-V --A--L-T---
```

FIGURE 3A and 3B

Light Chain

Antibody	*CDR1*
	24 25 26 27 a b c d 28 29 30 31 32 33 34
Murine monoclonal COL-1	RASKSVSASGYSYMH
Human VJI'CL	KS -Q- - LYNNKN- LA
HuCOL1	RA- K- -S ASGY S- MH
Minimally immunogenic variant	KS -Q- - S ASGYS- MH

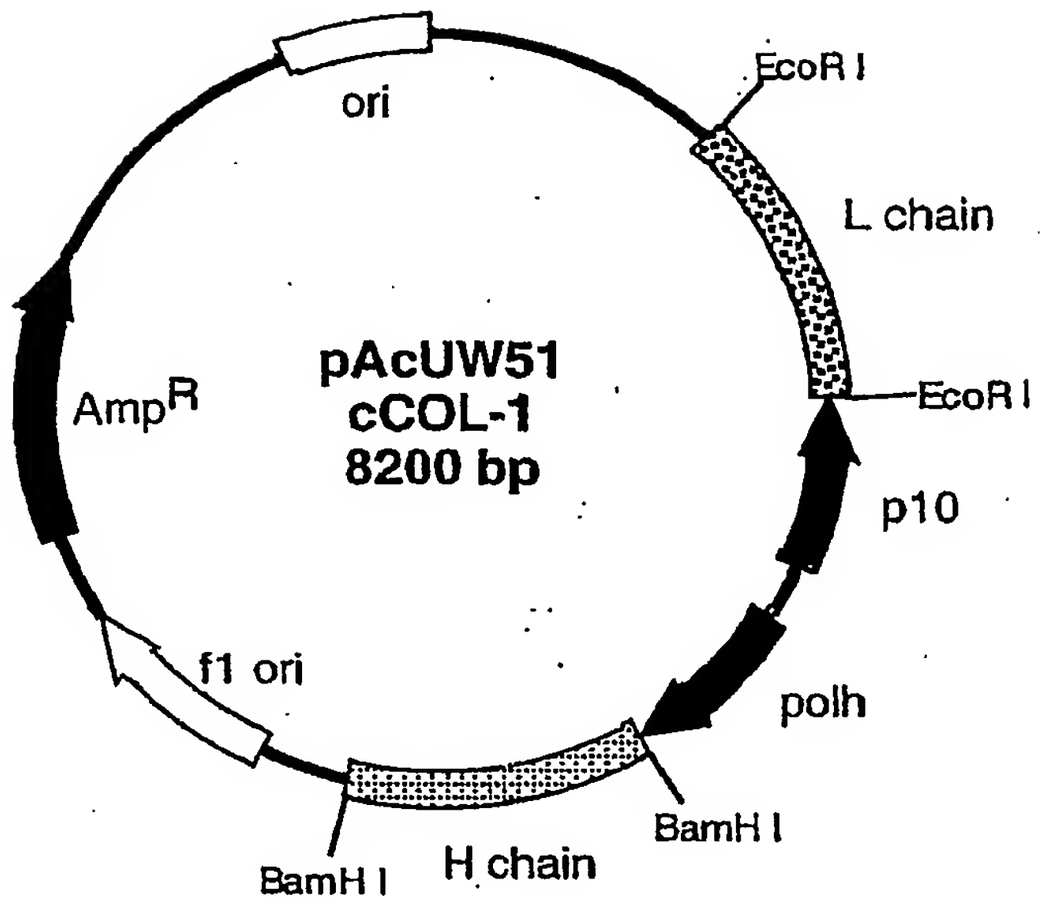
Heavy Chain

Antibody	*CDR2*
	50 5152 a 53 54 55 56 57 58 59 60 61 62 63 64 65
Murine monoclonal COL-1	WIDPENGDTEYAPKFQG
Human MO30	I- N- SGNS - N- - Q - - - -
HuCOL1	W-D-ENGD - E- - P - - - -
Minimally immunogenic variant	W-D-ENGD - E- - Q - - - -

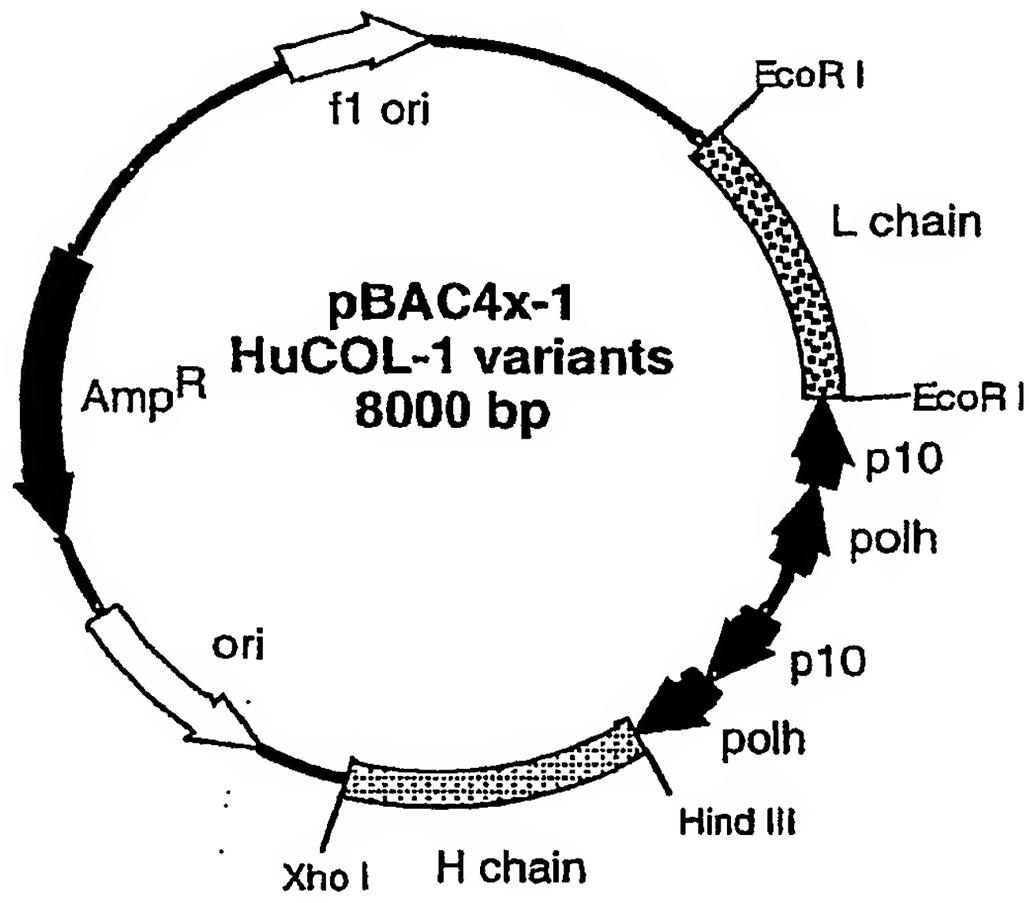
FIGURE 3C

FIGURE 4

A.


ori
EcoR I
L chain
EcoR I
p10
polh
BamH I
H chain
BamH I
f1 ori
Amp^R
pAcUW51
cCOL-1
8200 bp

B.


f1 ori
EcoR I
L chain
EcoR I
p10
polh
p10
polh
Hind III
H chain
Xho I
ori
Amp^R
pBAC4x-1
HuCOL-1 variants
8000 bp

A.

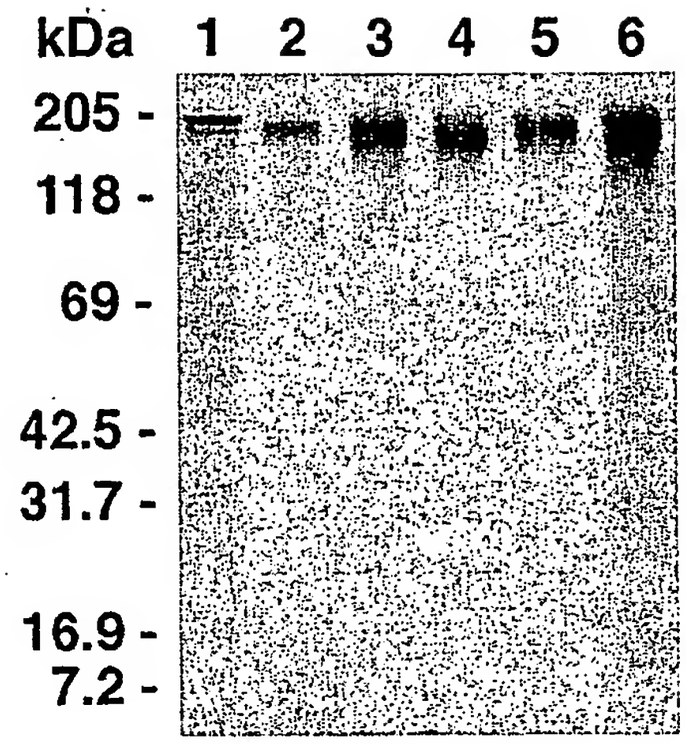


FIGURE 5A

B.

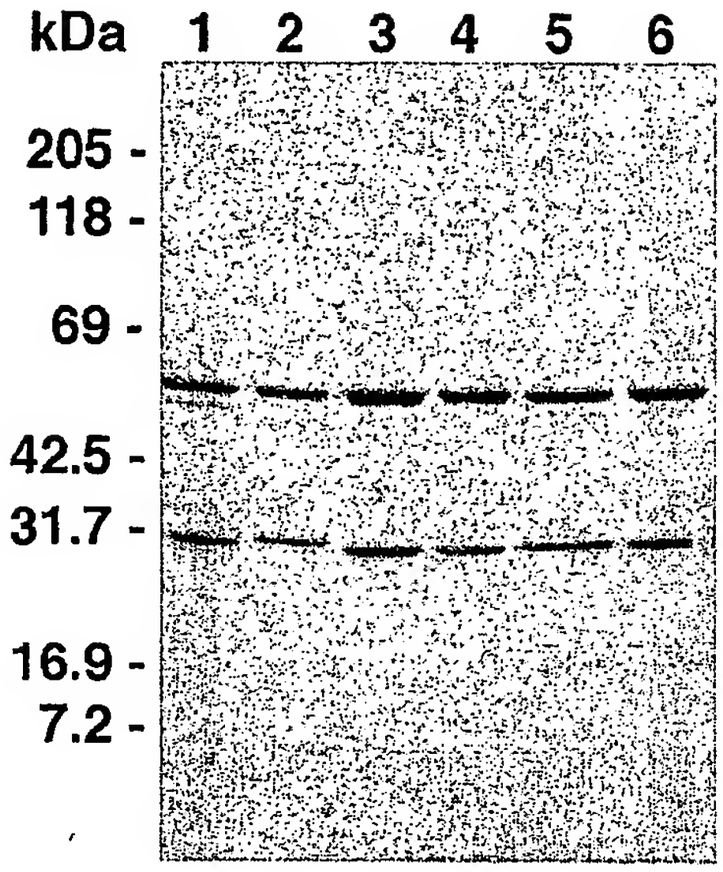


FIGURE 5B

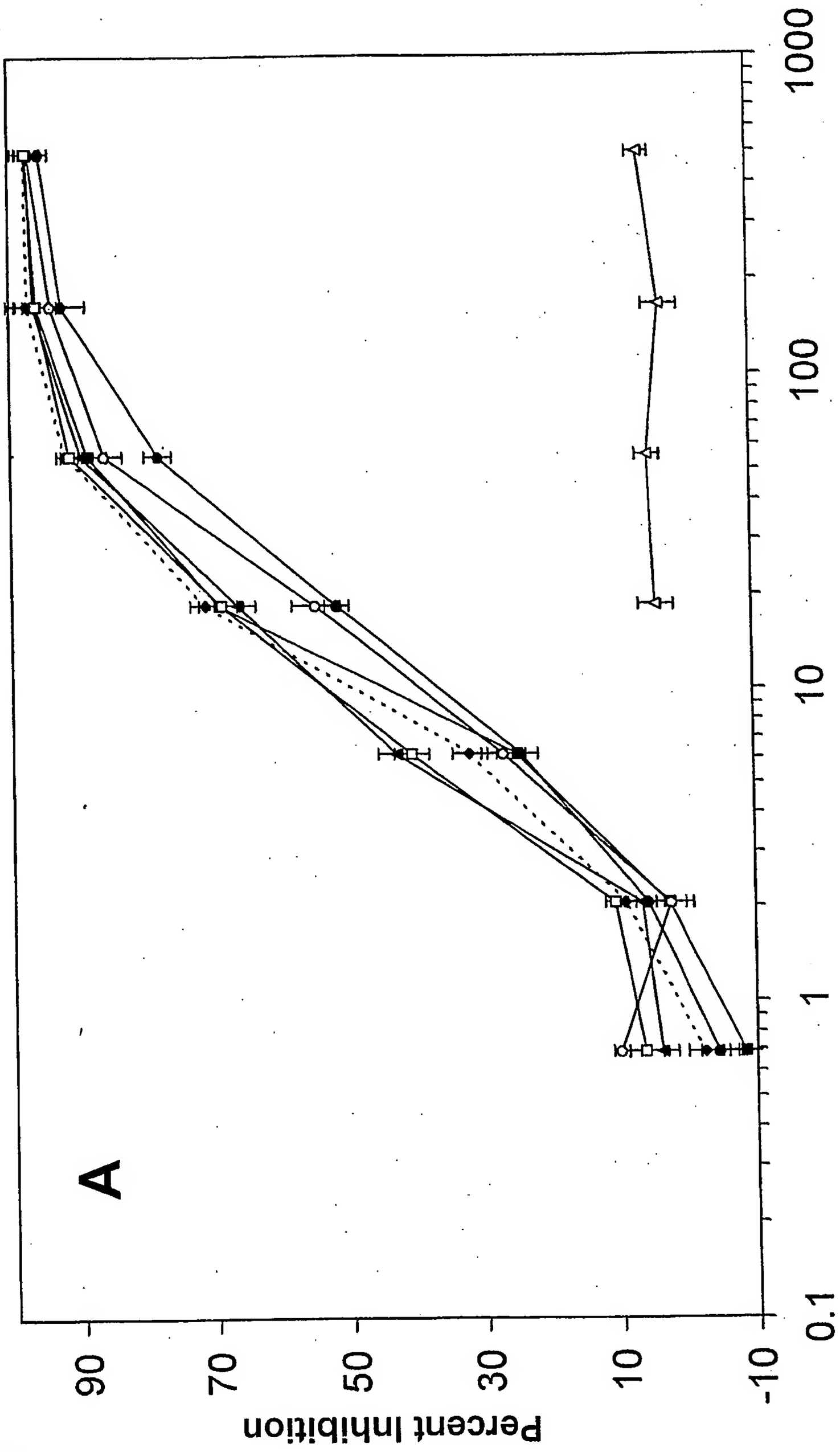

A
Percent Inhibition
90
70
50
30
10
-10
Competitor (ng/25ul)
0.1
1
10
100
1000

FIGURE 6A

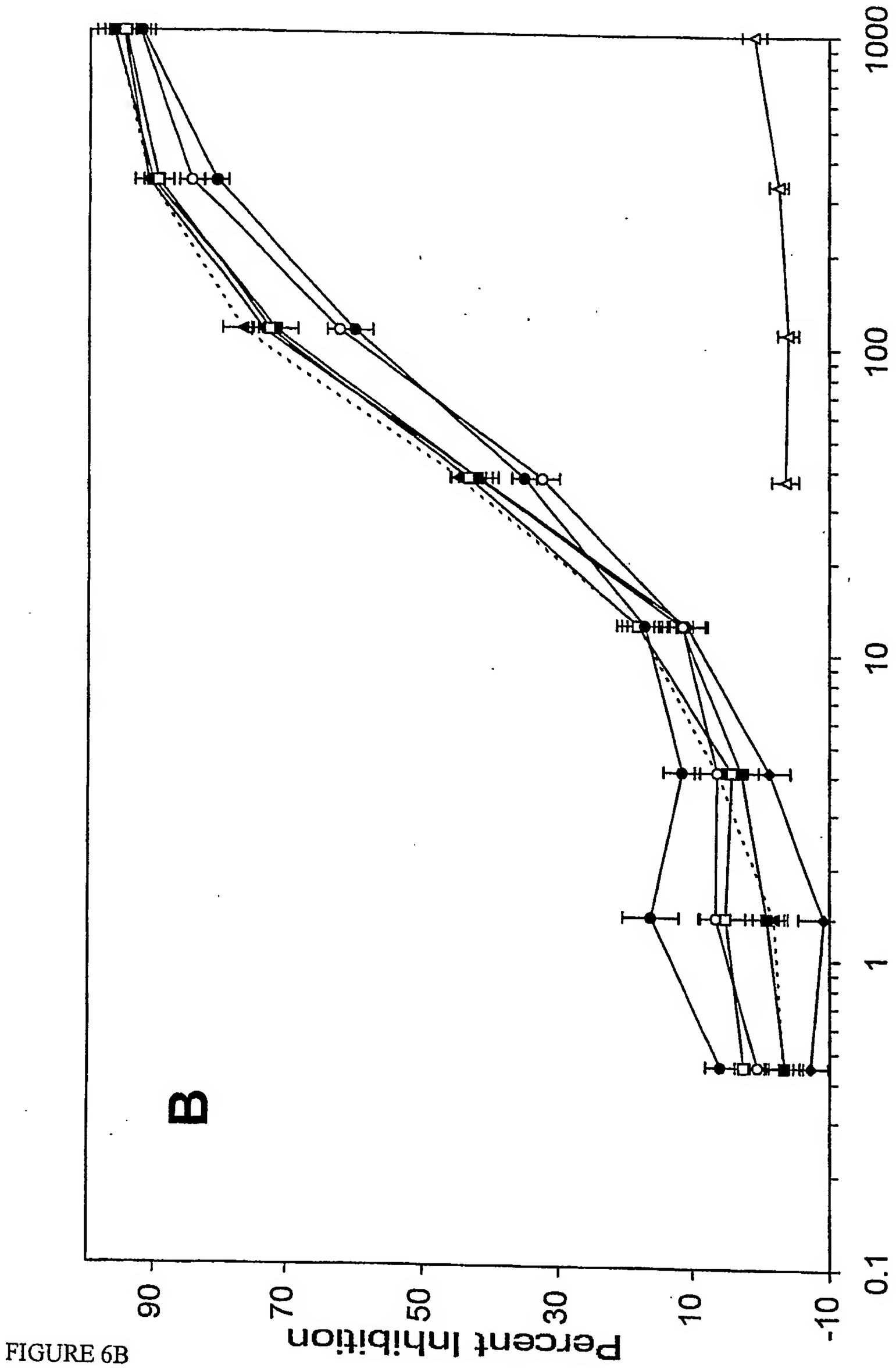
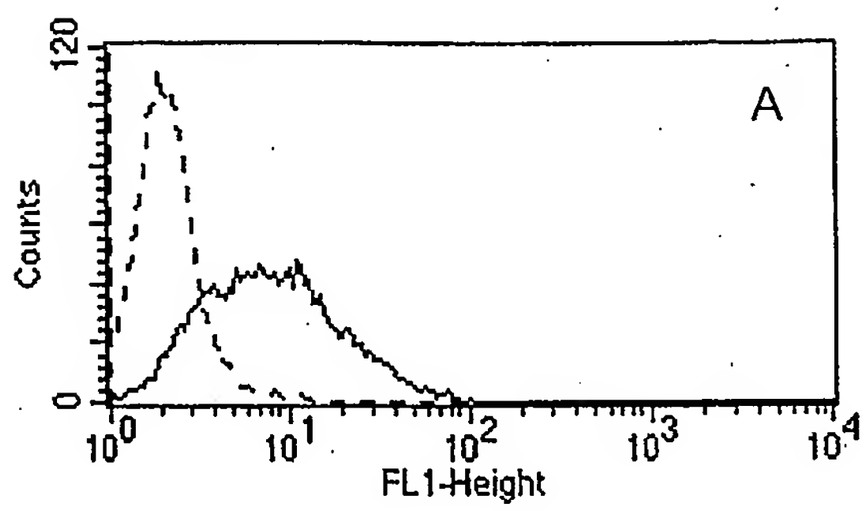
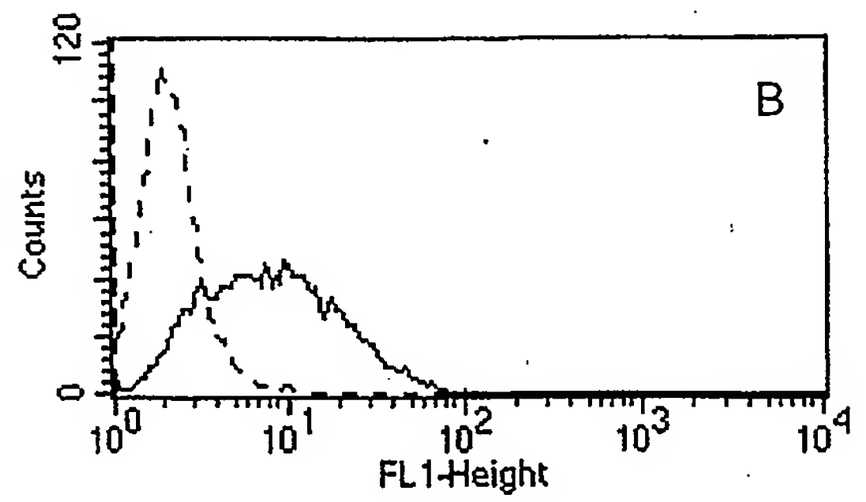
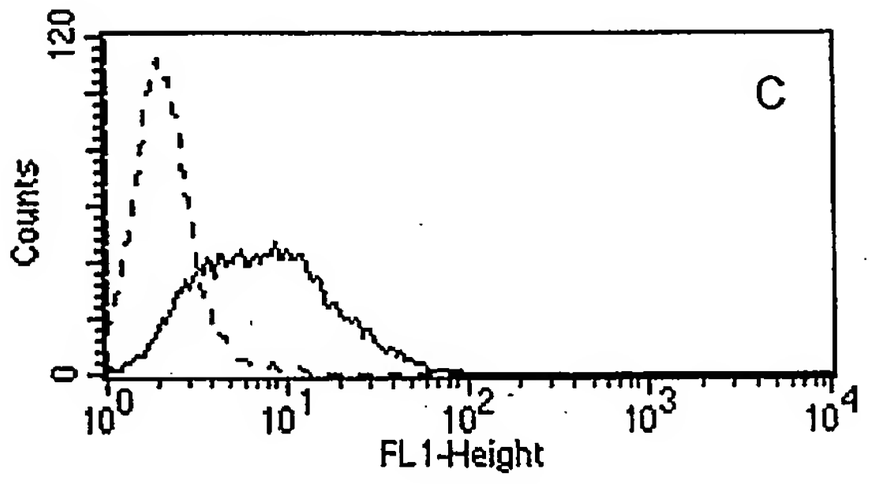
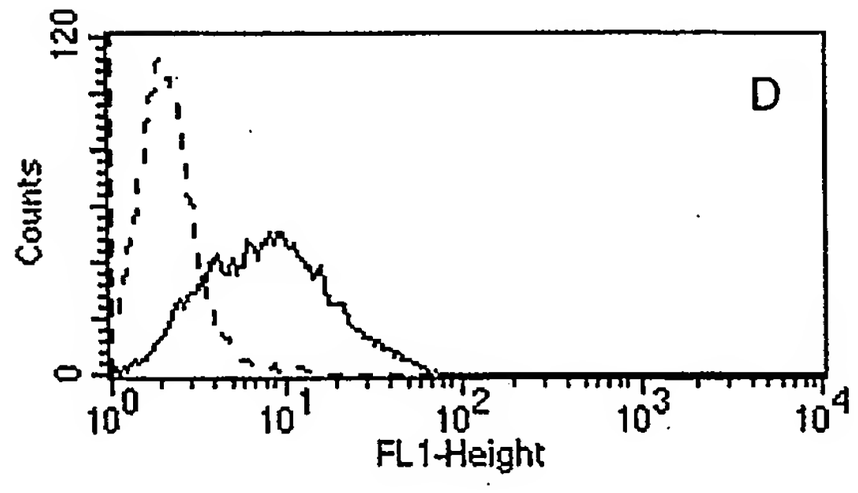

B
Percent Inhibition
90
70
50
30
10
-10
Competitor (ng/25ul)
0.1
1
10
100
1000

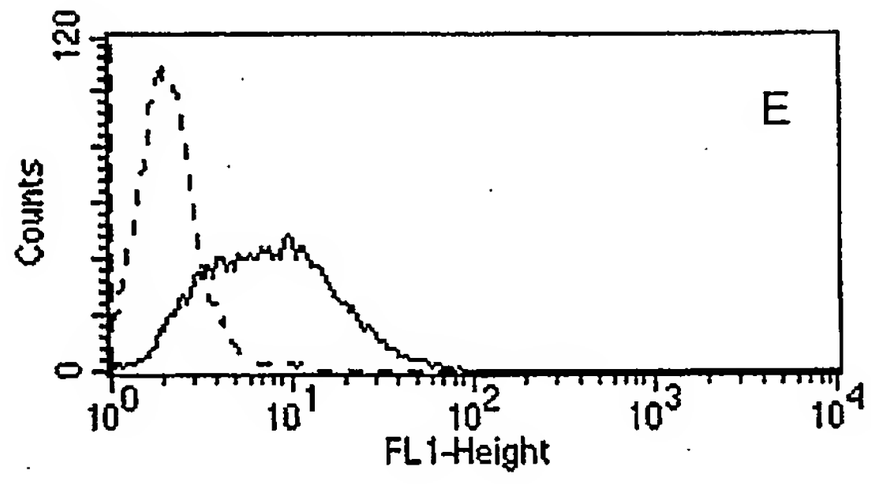
FIGURE 6B

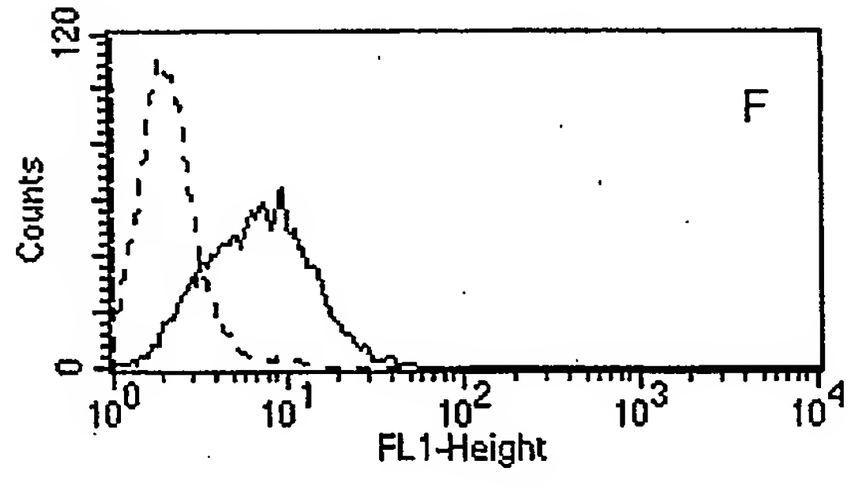

A
Counts
120
0
10^0
10^1
10^2
10^3
10^4
FL1-Height


B
Counts
120
0
10^0
10^1
10^2
10^3
10^4
FL1-Height


C
Counts
120
0
10^0
10^1
10^2
10^3
10^4
FL1-Height


D
Counts
120
0
10^0
10^1
10^2
10^3
10^4
FL1-Height


E
Counts
120
0
10^0
10^1
10^2
10^3
10^4
FL1-Height


F
Counts
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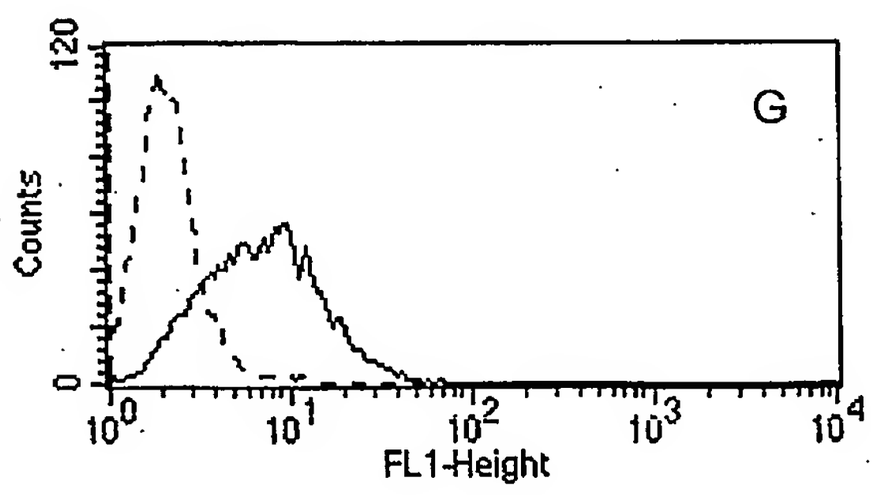
0
10^0
10^1
10^2
10^3
10^4
FL1-Height


G
Counts
120
0
10^0
10^1
10^2
10^3
10^4
FL1-Height

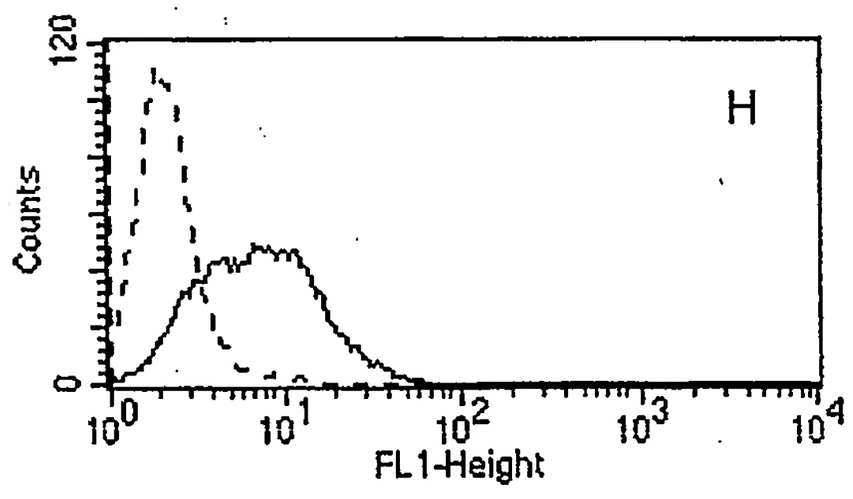

H
Counts
120
0
10^0
10^1
10^2
10^3
10^4
FL1-Height

FIGURE 7

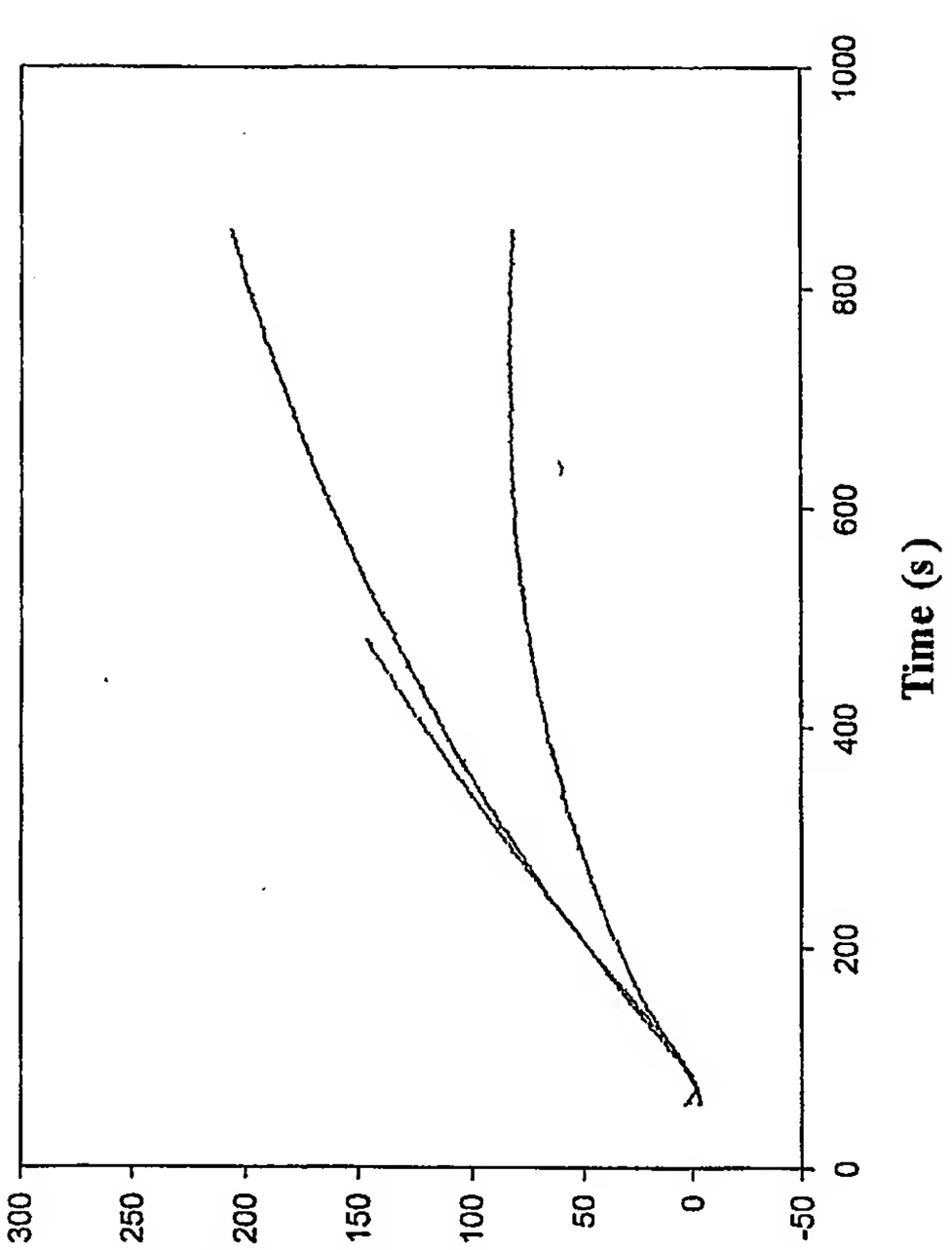

Response Difference (RU)
300
250
200
150
100
50
0
-50
0
200
400
600
800
1000
Time (s)

FIGURE 8

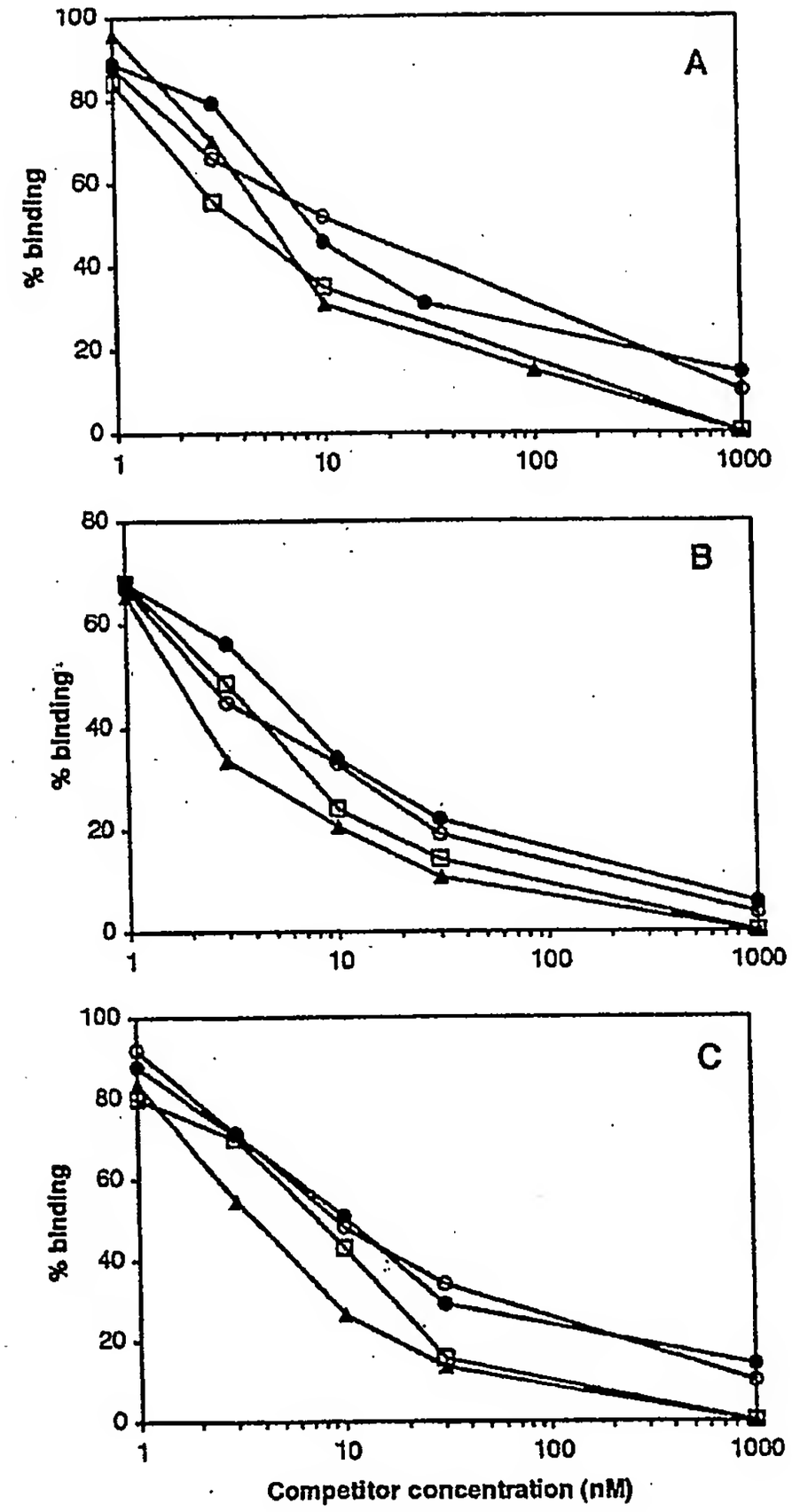

A
B
C
% binding
100
80
60
40
20
0
1
10
100
1000
Competitor concentration (nM)

FIGURE 9

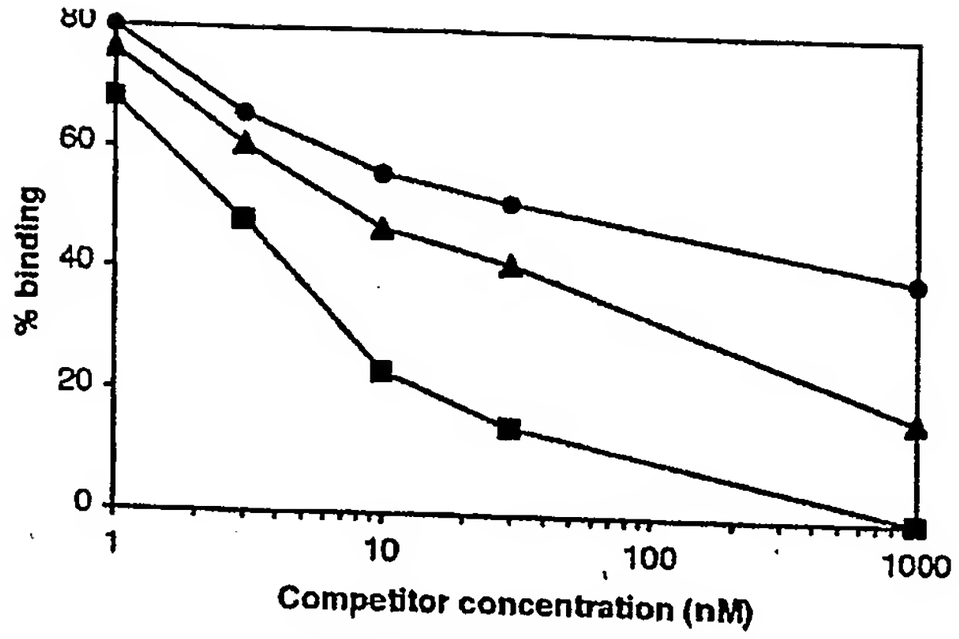

80
60
40
20
0
% binding
1
10
100
1000
Competitor concentration (nM)

FIGURE 10